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(54) Title: METHODS AND COMPOSITIONS FOR DETECTING AND TREATING MYCOBACTERIAL INFECTIONS USING AN inhA GENE

(57) Abstract

The embodiments of the invention are based upon the identification and characterization of genes that determine mycobacterial resistance to the antibiotic isoniazid (INH) and its analogs. These genes, termed *inhA*, encode a polypeptide, InhA, that is the target f action of mycobacteria for isoniazid. The sequences of wild-type INH-sensitive as well as allelic or mutant INH-resistant *inhA* genes and their operons are provided. Also provided are isolated InhA polypeptides of both the INH-resistant and INH-sensitive types.

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METHODS AND COMPOSITIONS FOR DETECTING AND TREATING MYCOBACTERIAL INFECTIONS USING AN inhA GENE

Statement of Government Interest

This invention was made with government support under NIH Grant No. A126170 and National Cooperative Drug Discovery Group Grant No. UO1A130189. As such, the United States government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a Continuation-in-Part of Application Serial No. 08/062,409 filed May 14, 1993, entitled USE OF GENES OF M. TUBERCULOSIS AND M. SMEGMATIS WHICH CONFER ISONIAZID RESISTANCE TO TREAT TUBERCULOSIS AND TO ASSESS DRUG RESISTANCE.

FIELD OF THE INVENTION

The invention relates to materials and methods used in the diagnosis and treatment of mycobacterial diseases, and more specifically to DNA sequence(s) associated with resistance to isoniazid and its analogs in mycobacteria, methods for isolating such sequences), and the use of such sequence(s) in human and animal medical practice.

BACKGROUND OF THE INVENTION

Tuberculosis caused by members of the M. tuberculosis complex including M. tuberculosis, M. bovis, and M. africanum remains the largest cause of human death

in the world from a single infectious disease, and is responsible for one in four avoidable adult deaths in developing countries. In addition, in 1990, there was a 10% increase in the incidence of tuberculosis in the United States. Further, M. bovis causes tuberculosis in a wide range of animals, and is a major cause of animal suffering and economic loss in animal industries.

Infection with drug-sensitive strains of the M. tuberculosis complex can be effectively cured with a combination of antibiotics, including isoniazid (isonicotinic acid hydrazide, INH), rifampicin, and pyrazinamide. INH was first reported to be active against M. tuberculosis in 1952, and particularly active against M. tuberculosis and M. bovis. However, mutants resistant to INH have emerged since then, and today such mutants account for as many as 26% of the clinical M. tuberculosis isolates in certain U.S. cities.

Some INH-resistant strains are associated with a loss of catalase activity, and deletions of the catalase-peroxidase gene (katG) correlate with INH resistance in certain M. tuberculosis isolates. Furthermore, transfer of the wild-type (wt) M. tuberculosis katG gene to INH-resistant M. smegmatis and M. tuberculosis confers INH sensitivity, suggesting that catalase-peroxidase activity is required for INH-sensitivity. However, in some studies only 10 to 25% of the INH-resistant isolates appear to be catalase negative, indicating that INH resistance can be due to other factors.

Drug resistance can be caused by many mechanisms, including mutations in the drug target that reduce the binding of the drug or mutations that lead to increased production of the target. The mechanism by which INH inhibits mycobacteria and its precise target of action are unknown. Biochemical evidence has suggested that both INH and ethionamide (ETH, a structural analog of INH) block

mycolic acid biosynthesis in mycobacteria. INH has been found to inhibit mycolic acid biosynthesis in cell-free extracts of mycobacteria, but the target protein has not been identified. In addition, in certain cases, low-level INH resistance correlates not with the loss of catalase activity but with the coacquisition of ETH resistance, suggesting that the two drugs may share a common target.

Because such a high percentage of the M. tuberculosis complex strains are resistant to INH, a great need exists to identify its targets of action, and thereby to devise rapid methods for identification of INH-resistant strains and methods of treating individuals for prevention and/or treatment of the disease associated with them.

SUMMARY OF THE INVENTION

This invention is based upon the discovery of a gene, inhA (also called ps5), that encodes an enzyme (InhA). InhA is the target of action of isoniazid in mycobacteria. Mutations within the inhA gene result in isoniazid resistance. Thus, the present invention provides isolated and recombinant polynucleotide sequences and polypeptides encoded therein that are associated with resistance to INH and its structural analogs in members of the genus mycobacteria, particularly those of the M. tuberculosis complex, including M. tuberculosis, M. africanum and M. bovis; the M. avium complex, including M. avium, M. intracellulare, M. scrofulaceum, and M. paratuberculosis; M. smegmatis. It also provides the allelic counterparts that are associated with INH sensitivity. The polynucleotides of the invention have many uses. For example, they are useful in assessing the susceptibility of various strains of the M. tuberculosis complex to isoniazid type antibiotics, as decoys and antisense oligonucleotides to prevent the expression of

polypeptides associated with isoniazid resistance, and for the expression of the polypeptides encoded therein. The polypeptides encoded in the polynucleotides and/or antibodies directed to them may also have use in immunoassays for the detection of INH-resistant strains, in the determination of whether an INH-type antibiotic may be effective against tuberculosis, and in the treatment of individuals for infection with these strains.

Accordingly, embodiments of the invention include the following.

An isolated wild-type gene which encodes an enzyme which is the target of action for isoniazid.

An isolated wild-type gene which encodes a polypeptide (InhA) which is the target of action for isoniazid (INH). These wild type genes also include those from M. tuberculosis, M. avium, M. smegmatis, and M. bovis.

An isolated mutant gene that encodes InhA wherein the mutant gene is associated with INH-resistance.

An isolated polynucleotide encoding an InhA polypeptide or fragment or variant thereof. These polynucleotides include recombinant expression vectors comprised of control sequences operably linked to a segment encoding the InhA polypeptide of fragment or variant thereof.

A host cell comprised of any of the aforementioned polynucleotides.

A method of treating an individual for infection caused by a member of the mycobacterial complex comprising:

(a) providing a composition comprised of a polynucleotide capable of inhibiting mRNA activity from an inhA operon of the infecting species and a suitable excipient; and

(b) administering a pharmacologically effective amount of said composition to the individual.

The above-mentioned method wherein the mode of

administration of the polynucleotides is oral, enteral, subcutaneous, intraperitoneal or intravenous.

A method of assessing susceptibility of a strain of mycobacteria in a biological sample to INH comprising:

(a) providing the mycobacterial DNA from the biological sample;

(b) amplifying a region of the inhA operon;

(c) determining whether a mutation exists within the inhA operon from the biological sample, the presence of the mutation indicating that said mycobacterial strain is resistant to INH.

The aforementioned method of wherein the amplification is by a polymerase chain reaction (PCR).

In addition, the aforementioned method further comprised of providing a comparable portion of wild-type INH-sensitive inhA operon from the mycobacteria, and the determination of whether a mutation exists in the biological sample is by comparison with the wild-type inhA operon.

The aforementioned method wherein determining whether a mutation exists is performed by single strand conformation polymorphism analysis.

A method of determining whether a drug is effective against mycobacterial infection comprising:

(a) providing isolated InhA;

(b) providing a candidate drug;

(c) mixing InhA with substrates for mycolic acid biosynthesis in the presence or absence of the candidate drug; and

(d) measuring the inhibition of biosynthesis of mycolic acid caused by the presence of the drug, if any.

A method of producing a tuberculosis-specific mycolic acid comprising adding purified InhA to substrates required for the biosynthesis of mycolic acid.

A method for producing a compound that inhibits

InhA activity comprising:

a. providing purified InhA;

b. determining the molecular structure of said InhA;

c. creating a compound with a similar molecular structure to INH; and

d. determining that said compound inhibits the biochemical activity of InhA.

An isolated InhA polypeptide or fragment or variant thereof.

A recombinant mycobacterial vaccine comprised of attenuated mutants selected from the group consisting of BCG, M. tuberculosis, and M. bovis, wherein the mutants are host cells containing a mutated inhA gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a table listing inhA genes from different mycobacteria that confer resistance to INH and ETH in M. smegmatis mc^2 155.

Figure 2, comprised of sheets 2A through 2F, presents a comparison of the coding strands of DNA sequences from M. bovis that confer resistance to INH and from M. tuberculosis and M. smegmatis that confer sensitivity to INH. The mutation in mc^2651 that causes INH-resistance is indicated by the arrow.

Figure 3 is a diagram of the subcloning strategy used to demonstrate that the isoniazid resistance phenotype is conferred by the inhA open reading frame.

Figure 4, comprised of sheets 4A and 4B, shows the alignment of the amino acid sequences of InhA proteins from M. tuberculosis H37R, M. bovis, M. bovis NZ, M. smegmatis mc^2155 and M. smegmatis mc^2651 with EnvM proteins from S. typhimurium and E. coli.

Figure 5 is a bar graph showing the results of cell-free assays of mycolic acid biosynthesis, and the effect of insertion of inhA genes on the effect of INH.

Figure 6 is a diagram of the allelic exchang experiment demonstrating that the p int mutation in the mc²651 inhA p lynucleotide results in INH-resistance, and the results obtained from the experiment.

Figure 7, comprised of sheets 7A-1 through 7A-2, 7B-1 through 7B-3, and 7C-1 through 7C-2, shows the nucleic acid sequence that includes the M. smegmatis inhA gene.

Figure 8, comprised of sheets 8A, 8B, and 8C, shows the nucleic acid sequence that includes the M. tuberculosis inhA gene.

Figure 9, comprised of sheets 9A, 9B, 9C and 9D, shows the nucleic acid sequence for pS5 and the amino acid sequence from two large open reading frames encompassed within it.

Figure 10 presents the amino acid sequence of a fragment encoded by nucleic acid residues 1256-2062 (ORF2) of the pS5 operon.

Figure 11 presents the amino acid sequence encoded by nucleic acid residues 494-1234 (ORF1) of the pS5 operon.

Figure 12, comprised of sheets 12A throufh 12C, presents the amino acid sequence of the M. bovis pS5 operon.

Figure 13 presents a restriction enzyme map of pYUB18 showing some significant features of the genome.

DETAILED DESCRIPTION OF THE INVENTION

The invention stems from the discovery of inhA, a gene that encodes a polypeptide that is a target for INH and ETH in members of the M. tuberculosis complex. Mutations of the gene render mycobacteria INH- and ETH-resistant. The gene and mutations within it were identified using a genetic strategy. Genomic libraries were constructed in shuttle c smid vectors from INH-

resistant mutants of M. smegmatis and M. bovis. Transferral of the libraries into wild type (i.e., INH-sensitive) M. smegmatis strains allowed the identification of clones that consistently conferred INH-resistance (shown in the table in Figure 1). The transformation of cosmids containing a cross-hybridizing DNA fragment from wt (INH-ETH-sensitive strains of M. smegmatis, M. tuberculosis, M. bovis, M. bovis BCG, and M. avium yielded clones that conferred INH-ETH resistance. These results were suggestive that overexpression of a putative target gene, inhA, on a multi-copy plasmid conferred an INH-resistance phenotype. This led to the conclusion that InhA, the gene product of inhA, is the target of action for INH in members of the M. tuberculosis complex. Moreover, the results showing that a 3 kb BamHI DNA fragment from the M. smegmatis cosmid that conferred INH-resistance strongly hybridized to all of eleven mycobacterial species tested demonstrated that the inhA gene is highly conserved among mycobacteria.

The DNA fragments that conferred INH-sensitivity to M. smegmatis and M. tuberculosis, as well as those that were isolated from mutant INH-resistant M. smegmatis and M. bovis strains were subjected to DNA sequencing. These DNA sequences are shown in Figure 2. Figure 2 presents the DNA sequences of the INH-resistant polynucleotide from M. bovis and the INH-sensitive polynucleotides of M. tuberculosis and M. smegmatis. Sequence analysis revealed two ORFs, encoding proteins of 29 and 32 kD. Subcloning analyses of the M. smegmatis fragment demonstrated that the ORF encoding the 29 kD protein was responsible for the INH-resistance phenotype, and was termed the inhA gene. In the M. bovis and M. tuberculosis genomes, it appears that the inhA genes are positioned such that they are subject to the same transcriptional control elements (including the promoter) as is ORF1, whereas the inhA gene has its own

promoter in the M. smegmatis genome.

The M. tuberculosis and M. smegmatis inhA gene products show 38 and 40% homologies to the envM gene product of S. typhimurium. In addition, in the M. smegmatis, M tuberculosis, and M. bovis genomes the inhA ORFs are preceded by another ORF that shares 40% identity with acetyl CoA reductases. The similarities of the inhA ORF and ORF1 to lipid biosynthetic genes are consistent with the hypothesis that INH inhibits an enzyme involved in mycolic acid biosynthesis.

Sequence analysis and comparison of inhA from the mutant INH-resistant and wt INH-sensitive strains of M. smegmatis (See Figure 4) and M. bovis revealed the presence of a single base pair difference that resulted in the amino acid substitution of an alanine for a serine at position 94 of the InhA protein. (See Figure 4.) As shown in the Examples, this difference caused the Inh-resistance phenotype.

Polynucleotides from M. smegmatis, M. tuberculosis, and M. bovis that encode InhA have been identified, isolated, cloned, sequenced and characterized. The nucleic acid sequences for these polynucleotides are shown in Figures 7, 8, and 9 respectively. Figure 9 also shows the amino acids encoded in the polynucleotide.

A comparison of the sequences for M. tuberculosis inhA and M. bovis inhA shows that the inhA gene from INH-sensitive M. tuberculosis and INH-sensitive M. bovis are identical. Given that the mutation of Ser to Ala conferring INH-resistance is conserved in M. smegmatis and M. bovis phenotypes, it can be anticipated that other INH-resistant isolates will be found that are due to mutations in the inhA operon. For example, INH-resistance may also be due to missense mutations in the coding region of inh, or to mutations that cause the overexpression of InhA (e.g., mutations in the regulatory regions of the operon,

and/or duplications that allow overexpression).

The discovery of inhA genes and operons of the mycobacterial complex that confer INH-resistance allows for the preparation and use of compositions and methods useful in the diagnosis and treatment of pathogenic states resulting from infection with these microorganisms, and particularly with INH-resistant strains.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition (1989), OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait Ed., 1984), the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.M. Miller and M.P. Calos eds. 1987), HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, (D.M. Weir and C.C. Blackwell, Eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Siedman, J.A. Smith, and K. Struhl, eds., 1987), and CURRENT PROTOCOLS IN IMMUNOLOGY (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober, eds., 1991).

As used herein the term "target of action for isoniazid" refers to a polypeptide, InhA, encoded in an inhA operon of mycobacteria, and preferably in members of the mycobacterial complex.

As used herein, the term "inhA gene" refers to a polynucleotide that encodes a polypeptide that is present in mycobacteria, wherein the polypeptide has substantial amino acid homology and equivalent function to the InhA proteins of M. tuberculosis, M. bovis, or M. smegmatis; amino acid sequences of variants of these InhA proteins are shown in Figure 4. In this context substantial amino acid

homology means at least about 60% homology, generally at least about 70% homology, even more generally at least about 80% homology, and at times at least about 90% homology to any of the indicated polypeptides.

As used herein the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art (*e.g.*, Sambrook, *et al.*), methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*), those containing pendant moieties, such as, for example, proteins (including for *e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, *etc.*), those with intercalators (*e.g.*, acridine, psoralen, *etc.*), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, *etc.*), those containing alkylators, those with modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*), as well as unmodified forms of the polynucleotide.

The invention includes as an embodiment an isolated polynucleotide comprised of a sequence encoding a polypeptide associated with isoniazid (INH) resistance in mycobacteria or active fragment thereof. These isolated polynucleotides contain less than about 50%, preferably less than about 70%, and more preferably less than about 90% of the chromosomal genetic material with which the sequence encoding the polypeptide is usually associated in nature. An isolated polynucleotide "consisting essentially of" a sequence encoding an isoniazid resistance associated polypeptide lacks other sequences encoding other

polypeptides derived from the mycobacterial chromosome.

As used herein "isoniazid" ("INH") refers to isoniazid and analogs thereof that inhibit mycobacterial replication by inhibiting the activity of the same polypeptide(s) INH inhibits, for example, ethonamide (ETH).

The invention also includes as embodiments recombinant polynucleotides containing a region encoding inhA gene products for mycobacteria. The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature; or (2) is linked to a polynucleotide other than that to which it is linked in nature; or (3) does not occur in nature.

A purified or recombinant polynucleotide comprised of a sequence encoding InhA of mycobacteria or variant or active fragment thereof, may be prepared by any technique known to those of skill in the art using the polynucleotide sequences provided herein. For example, they can be prepared by isolating the polynucleotides from a natural source, or by chemical synthesis, or by synthesis using recombinant DNA techniques.

It is contemplated that the sequence encoding an InhA encodes a polypeptide that is associated with isoniazid resistance or sensitivity in mycobacteria, and that allelic variations of the sequences, some of which are shown in the Figures are contemplated herein. The term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the

definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), polypeptides with substituted linkages, as well as the modifications known in the art, both naturally occurring and non-naturally occurring.

Also contemplated within the invention are cloning vectors and expression vectors comprised of a sequence encoding InhA or variant or fragment thereof. Suitable cloning vectors may be constructed according to standard techniques, or may be selected from the large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self replicate, may possess a single target for a restriction endonuclease, and may carry genes for a readily selectable marker (e.g., antibiotic resistance or sensitivity markers). Suitable examples include plasmids and bacterial viruses, e.g., PUC18, mp18, mp19, PBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors (e.g., pSA3 and pAT28. Preferred vectors include pBluescript IIks (Stratagene), and pYUB18.

Expression vectors generally are replicable polynucleotide constructs that encode a polypeptide operably linked to suitable transcriptional and translational regulatory elements. Examples of regulatory elements usually included in expression vectors are promoters, enhancers, ribosomal binding sites, and transcription and translation initiation and termination sequences. The regulatory elements employed in the expression vectors containing a polynucleotide encoding InhA or an active fragment would be functional in the host cell used for expression. It is also contemplated that the regulatory sequences may be derived from the inhA operon; thus, a promoter or terminator sequence may be homologous (i.e., from mycobacteria) to the coding sequence.

The invention also includes recombinant host cells comprised of any of the above described polynucleotides that contain a sequence encoding an InhA polypeptide of mycobacteria. The polynucleotides may be inserted into the host cell by any means known in the art. As used herein, "recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. Hosts which may be used include prokaryotic cells (e.g., bacterial cells such as E. coli, mycobacteria, and the like) and eukaryotic cells (e.g., fungal cells, insect cells, animal cells, and plant cells, and the like). Prokaryotic cells are generally preferred, and E. coli and M. smegmatis are particularly suitable. Of the latter, mc^2155 is particularly preferred.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome.

The polynucleotides comprised of sequences encoding InhA are of use in the detection of INH-resistant forms of mycobacteria in biological samples. As used herein, a "biological sample" refers to a sample of tissue

labelled, for example with radioactive isotopes. Usual isotopes include, for example ^{32}P and ^{33}P. The probes are capable of hybridizing to the genetic elements associated with INH-resistance. Preferably, the probes are specific for sequences that encode the INH-resistance gene. By way of example, the probe may be the entire nucleotide sequence depicted in Figure 12. However, shorter probes are preferred.

For use of such probes as diagnostics, the biological sample to be analyzed, such as blood or serum, may be treated, if desired, to extract the nucleic acids contained therein. The resulting nucleic acid from the sample may be subjected to gel electrophoresis or other size separation techniques; alternatively, the nucleic acid sample may be dot blotted without size separation. The probes are usually labeled. Suitable labels, and methods for labeling probes are known in the art, and include, for example, radioactive labels incorporated by nick translation or kinasing, biotin, fluorescent probes, and chemiluminescent probes. The nucleic acids extracted from the sample are then treated with the labeled probe under hybridization conditions of suitable stringencies. The probes can be made completely complementary to the allelic form of polynucleotide that has been targeted. With this goal, high stringency conditions usually are desirable in order to prevent false positives. The stringency of hybridization is determined by a number of factors during hybridization and during the washing procedure, including temperature, ionic strength, length of time, and concentration of formamide. These factors are outlined in, for example, Maniatis, T. (1982).

It may be desirable to use amplification techniques in hybridization assays. Such techniques are known in the art and include, for example, the polymerase chain reaction (PCR) technique described which is by Saiki

or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively infected cells, recombinant cells, and cell components). As used herein, the term "clinical sample" is synonymous with "biological sample".

The term "individual" as used herein refers to vertebrates, particularly members of the mammalian or avian species, and includes but is not limited to domestic animals, sports animals, and primates, including humans.

Using the disclosed portions of the isolated polynucleotides encoding InhA as a basis, oligomers of approximately 8 nucleotides or more can be prepared, either by excision from recombinant polynucleotides or synthetically, which hybridize with the mycobacterial sequences in the plasmids and are useful in identification of the INH-resistant and INH-sensitive mycobacteria. The probes are a length which allows the detection of the InhA encoding sequences by hybridization. While 6-8 nucleotides may be a workable length, sequences of 10-12 nucleotides are preferred, and about 20 nucleotides appears optimal. These probes can be prepared using routine methods, including automated oligonucleotide synthetic methods. For use as probes, complete complementarity is desirable, though it may be unnecessary as the length of the fragment is increased.

Thus, a polynucleotide comprising all or part of the nucleic acid sequences of an inhA operon, and particularly an inhA gene may be used as probes for identifying nucleic acids which code for polynucleotides associated with INH-resistance. The probes may be

et al. (1986), by Mullis, U.S. Patent No. 4,683,195, and by Mullis et al. U.S. Patent No. 4,683,202. This technique may be used in conjunction with other techniques, for example, in single-strand conformation polymorphism analysis (see infra., in the Examples).

The probes can be packaged into diagnostic kits. Diagnostic kits include the probe DNA, which may be labeled; alternatively, the probe DNA may be unlabeled and the ingredients for labeling may be included in the kit in separate containers. The kit may also contain other suitably packaged reagents and materials needed for the particular hybridization protocol, for example, standards, as well as instructions for conducting the test. If the kit is to be used for an assay system which includes PCR technology it may also include primers for the PCR reaction.

The inhA gene sequence and polypeptides encoded therein may also be used for screening for drugs against mycobacteria, particularly members of the mycobacterial complex, and more particularly M. tuberculosis and M. bovis. For example, it can be used to express the INH-resistant and INH-sensitive polypeptides encoded in the allelic forms of inhA. Utilizing these polypeptides in vitro assays, one could monitor the effect of candidate drugs on mycolic acid biosynthesis. Drugs that inhibit mycolic acid biosynthesis are candidates for therapy of mycobacterial diseases. Drugs that may be tested for effectiveness in this type of system include INH, ETH, rifampicin, streptomycin, ethambutol, ciprofloxacin, novobiocin and cyanide.

The inhA operon sequences may also be used to design polynucleotides that can be used for treatment of mycobacterial infections, including those caused by M. tuberculosis, M. avium, M. smegmatis, and M. bovis. One method of treating a mycobacterial infection utilizing the

InhA gene is by providing antisense polynucleotides or triplex forming polynucleotides which can be used to inhibit the transcription or translation of MRNA from the inhA operon, for example antisense polynucleotides, triplex forming polynucleotides, decoys, and ribozymes. Thus, these types of polynucleotides are also included within the invention. These polynucleotides may be prepared by a variety of techniques known in the art, including chemical synthesis and recombinant technology. After preparation they can then be administered, either alone or in combination with other compositions to treat mycobacterial infections, including tuberculosis. The compositions containing these polynucleotides may also include suitable excipients.

The sequence of inhA can also be used to assess the susceptibility of various strains of mycobacteria, and particularly of M. tuberculosis or M. bovis, in a clinical sample to INH. This susceptibility comparison is based upon the detection of a mutant allele as compared to the wild-type inhA allele that is INH-sensitive. Procedures to perform this type of assessment will be readily evident to those of skill in the art. For example, one procedure to perform this assessment is described in the Examples, and is based upon isolation of the chromosomal DNA of the bacterium, amplification of the inhA region by PCR using primers specific for the region (based upon the inhA sequences provided herein, and determination whether a mutation exists in the isolated DNA by the method of single strand conformation polymorphism analysis.

In addition, compounds which block the activity of InhA polypeptides (which may be enzymes) can be prepared utilizing the sequence information of inhA. This is performed by overexpressing InhA, purifying the polypeptide, and then performing X-ray crystallography on the purified InhA polypeptide to obtain its molecular

structure. Next, compounds are created which have similar molecular structures to all or portions of the polypeptide. The compounds are then combined with the polypeptide and attached thereto so as to block the biochemical activity of the InhA polypeptide.

The inhA polynucleotides may also be used produce or improve live attenuated or killed tuberculosis vaccines. For example, a tuberculosis strain which contains a mutated inhA can be administered in vaccine form to eliminate INH-resistance which is typically conferred by mutant inhA. In addition, mutated inhA genes may be added to BCG or M. tuberculosis vaccines to provide attenuated mutant tuberculosis vaccines. These vaccines may be used to treat and prevent a wide variety of diseases, including tuberculosis, AIDS, leprosy, polio, malaria and tetanus.

The polypeptides of the invention include those encoded in allelic variants of inhA, some of which are shown in the Figures herein, and are in purified or recombinant form. These polypeptides include fragments of the entire polypeptides encoded in the ORFs, particularly fragments that exhibit activity in mycolic acid biosynthesis. In addition, polypeptides of the invention include variants of InhA which differ from the native amino acid sequences by the insertion, substitution, or deletion of one or more amino acids. These variants may be prepared chemically, or by alteration of the polynucleotide sequence encoding InhA, using techniques known in the art, for example, by site-specific primer directed mutagenesis. These polypeptides can be purified by any means known in the art, including, for example freeze-thaw extraction, salt fractionation, column chromatography, affinity chromatography and the like.

The polypeptides of the invention may find use as therapeutic agents for treatment of mycobacterial infection. "Treatment" as used herein refers to

prophylaxis and/or therapy.

The InhA polypeptides can be prepared as discrete entities or incorporated into a larger polypeptide, and may find use as described herein. The immunogenicity of the epitopes of InhA may also be enhanced by preparing them in mammalian or yeast systems fused with or assembled with particle-forming proteins such as, for example, that associated with hepatitis B surface antigen. See, e.g., U.S. Pat. No. 4,722,840. Vaccines may be prepared from one or more immunogenic polypeptides derived from InhA.

The preparation of vaccines which contain an immunogenic polypeptide(s) as active ingredients, is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria,

monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an InhA antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations or formulations suitable for distribution as aerosols. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium,

calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5 micrograms to 250 micrograms of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reenforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the vaccine containing the immunogenic InhA antigen(s) may be administered in conjunction with other immunoregulatory agents, for example, immune globulins, as well as antibiotics.

The InhA antigens may be used for the preparation of antibodies. The immunogenic polypeptides prepared as described above are used to produce antibodies, including polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an immunogenic polypeptide

bearing an InhA epitope(s). Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an InhA epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987).

Monoclonal antibodies directed against InhA epitopes can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al. (1980); Hammerling et al. (1981); Kennett et al. (1980); see also, U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against InhA epitopes can be screened for various properties; i.e., for isotype, epitope affinity, etc.

Antibodies, both monoclonal and polyclonal, which are directed against InhA epitopes are particularly useful in diagnosis, and those which are neutralizing may be useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies.

Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired. See, for example, Nisonoff, A., et al. (1981) and Dreesman et al. (1985). Techniques for raising anti-idiotype antibodies are known in the art. See, for example, Grzych (1985), MacNamara et al. (1984), and Uytdehaag et al. (1985). These anti-idiotype antibodies may also be useful

for treatment, vaccination and/or diagnosis of mycobacterial infections, as well as for an elucidation of the immunogenic regions of InhA antigens.

Both the InhA polypeptides and anti-InhA antibodies are useful in immunoassays to detect presence of antibodies to mycobacteria, or the presence of the InhA antigens, and particularly the presence of INH-resistant InhA in biological samples. Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. The immunoassay will utilize at least one epitope derived from InhA. In one embodiment, the immunoassay uses a combination of epitopes derived from InhA. These epitopes may be derived from the same or from different bacterial polypeptides, and may be in separate recombinant or natural polypeptides, or together in the same recombinant polypeptides. An immunoassay may use, for example, a monoclonal antibody directed towards an InhA epitope(s), a combination of monoclonal antibodies directed towards epitopes of one mycobacterial antigen, monoclonal antibodies directed towards epitopes of different mycobacterial antigens, polyclonal antibodies directed towards the same antigen, or polyclonal antibodies directed towards different antigens. Protocols may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Typically, an immunoassay for an anti-InhA antibody(s) will involve selecting and preparing the test

sample suspected of containing the antibodies, such as a biological sample, then incubating it with an antigenic (i.e., epitope-containing) InhA polypeptide(s) under conditions that allow antigen-antibody complexes to form, and then detecting the formation of such complexes. Suitable incubation conditions are well known in the art. The immunoassay may be, without limitations, in a heterogenous or in a homogeneous format, and of a standard or competitive type.

In a heterogeneous format, the polypeptide is typically bound to a solid support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidine fluoride (known as Immulon), diazotized paper, nylon membranes, activated beads, and Protein A beads. For example, Dynatech Immulon ^{1} or Immulon ^{2} microtiter plates or 0.25 inch polysterene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptide is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are known in the art.

Complexes formed comprising anti-InhA antibody (or, in the case of competetive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For example, unlabeled anti-InhA antibodies in the complex may be detected using a conjugate of antixenogeneic Ig complexed with a label, (e.g., an enzyme label).

In immunoassays where InhA polypeptides are the analyte, the test sample, typically a biological sample, is incubated with anti-InhA antibodies under conditions that

allow the formation of antigen-antibody complexes. It may be desirable to treat the biological sample to release putative bacterial components prior to testing. Various formats can be employed. For example, a "sandwich assay" may be employed, where antibody bound to a solid support is incubated with the test sample; washed; incubated with a second, labeled antibody to the analyte, and the support is washed again. Analyte is detected by determining if the second antibody is bound to the support. In a competitive format, which can be either heterogeneous or homogeneous, a test sample is usually incubated with antibody and a labeled, competing antigen is also incubated, either sequentially or simultaneously. These and other formats are well known in the art.

The following examples are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

EXAMPLES

Example 1

Selection of INH-Resistant M. bovis Strains

In order to select M. bovis INH-resistant strains, a virulent wild-type New Zealand strain of M. bovis was cloned by four serial passages using a combination of liquid Tween albumin broth (TAB) and 7H10 pyruvate agar culture media. A single colony of M. bovis was inoculated into TAB and incubated until visible growth was apparent. An appropriate dilution of the bacterial suspension in TAB was plated onto the agar media to obtain discrete colonies. After incubation, a single colony was picked and inoculated into TAB and the cloning procedure

was repeated. After four cloning cycles a G4 strain was obtained. INH-resistant strains were obtained by growing the GI strain in liquid TAB media containing differing concentrations of INH. After incubation, the strain that had luxuriant growth in the highest concentration of INH was inoculated onto INH-containing solid media and incubated for growth. A colony was picked, used as inoculum for INH-containing TAB, and incubated under growth conditions. When visible growth was apparent, the medium was used to inoculate liquid TAB media containing INH, and the inoculated medium was allowed to incubate under growth conditions. Aliquots of the culture were then grown again in liquid TAB media containing increased differing concentrations of INH, and cloning of a colony from a strain that had luxuriant growth in the highest concentration of INH was repeated. This selection procedure was repeated and a series of clones of M. bovis with increasing resistance to isoniazid were obtained. The last strain selected, G4/100, was resistant to 100 μg/ml of INH.

Example 2

Isolation of INH-resistant Clones from a Cosmid Library prepared from an INH-Resistant Strain

A cosmid library from strain G4/100 was prepared in the shuttle vector pYUB18. Plasmid pYUB18 is a multicopy E. coli-mycobacteria shuttle cosmid that contains a selectable kanamycin gene and a cos site (J.T. Beslile et al., J. Bacteriol. 173, 6991 (1991); S.B. Snapper et al., Mol. Microbiol. 4:1911 (1990); W.R. Jacobs et al., Methods Enzymol. 204:537 (1991)). A restriction enzyme map of pYUB18 showing some significant features of the genome is shown in Figure 13.

The cosmid library was prepared as follows using standard techniques. Chromosomal DNA was purified from

G4/100, and subjected to partial digestion with Sau3A1; fragments between about 30-50 kb were purified by sucrose gradient purification and ligated to linearized pYUB18. Resulting cosmids were packaged into λ-phage using a commercial kit (Gigapack Gold Stratagene) according to the manufacturer's directions, and transfected into E. coli; approximately 5000 colonies were obtained. The colonies were pooled and the plasmids amplified, using standard plasmid preparation techniques.

The cosmid library was then transformed into M. smegmatis strain mc^2 155 by electroporation. Transformants were selected by growth on medium containing kanamycin. Approximately 1200 kanamycin resistant clones were patched onto media containing INH. Four INH resistant clones were identified.

Example 3

Isolation and Sequencing of pS5

In order to obtain a plasmid containing mycobacterial genetic material that conferred INH-resistance, the plasmids were extracted from the transformants. Cultures of M. smegmatis (5ml) were incubated with cycloserine and ampicillin for 3 hours before harvest. The cells were pelleted and resuspended in 0.25 ml of 40 mM Tris acetate, 2 mM EDTA, pH 7.9. To this, 0.5 ml of lysing solution was added (50 mM Tris, 3% sodium dodecylsulfate (SDS)) and the solution was mixed for 30 minutes. The sample was heated to 60°C for 20 minutes, cooled for 10 minutes and the DNA was extracted by adding 0.8 ml of phenol (containing 50 mM NaCl). This was centrifuged and the upper layer containing the DNA was removed. To precipitate the DNA, a half volume of 7.5 M ammonium acetate was added, incubated on ice for 30 minutes and then centrifuged for 30 minutes. The DNA was resuspended in 10 mM Tris, 1 mM EDTA.

The smallest plasmid obtained which conferred an Inh-resistance phenotype on M. smegmatis was 2.3 kb in size and was designated pS5.

The sequence of pS5 was obtained as follows. pS5 was cloned into the vector pBluescript II KS+ (Stratagene, California). This vector contains the T3 and T7 promoters which were used for the sequencing. Sequencing was carried out using the dsDNA cycle sequencing system from GIBCO BRL, Life Technologies, according to the manufacturer's directions. The radioactive labelled nucleotide was either [γ-^{32}P] ATP or [γ-^{33}P] ATP, available from Amersham. The sequencing program used was GCG, Sequence analysis software package. The nucleic acid sequence for pS5 and the amino acid sequence from two large open reading frames encompassed within it are shown in Figure 9. Figure 10 presents the amino acid sequence of a fragment encoded by nucleic acid residues 1256-2062 (the InhA gene) of the pS5 operon. Figure 11 presents the amino sequence of a fragment encoded by nucleic acid residues 494-1234 of the pS5 operon. Figure 12 presents the nucleic acid sequence of the pS5 M. bovis NZ operon.

Example 4

Determination of Catalase Activity in an INH-Resistant Strain

Catalase activity of an INH-sensitive strain of M. bovis was determined. The enzyme was first isolated from the strain by pelleting a culture of M. bovis, resuspending it in 50 mM potassium phosphate buffer, pH 7, and adding it to a tube containing 0.5 g zirconium beads (Biospecs products), and vortexing for 5 min. The sample was centrifuged briefly, the supernatant collected and diluted to 4 ml with 50 mM potassium phosphate buffer, and filter sterilized through 0.22 μm filters.

Catalase catalyzes the conversion of H_2O_2 to H_2O and O_2. Catalase activity was assayed by incubating an aliquot of supernatant, prepared as above, with 3 μm H_2O_2 in a total volume of 3 ml for 5 minutes. The reaction was stopped by adding 1.5 ml of titanium tetrachloride reagent (1.5 mg/ml $TiCl_4$ in 4.5 M H_2SO_4). The absorbance was read at 410 nm and the catalase activity was calculated using a standard curve of the amount of hydrogen peroxide versus wavelength at 410 nm; the activity was expressed as μmol/min/mg protein.

Catalase activity of G4/100, G4 and another virulent M. bovis strain were also determined using the above-described procedure. The G4 strain and other virulent M. bovis strains contained similar levels of catalase activity. Catalase activity was not detected in the G4/100 strain.

To demonstrate that the development of INH-resistance in G4/100 was not due entirely to loss of catalase activity, the plasmid pS5 was electroporated into G5 to produce G4(S5). G4(S5) grew on media containing a level of INH that prevented growth of G4. Using the method described above, catalase activity was tested in both G4 and G4(S5). G4(S5) which is INH-resistant, and G4 which is INH-sensitive, both showed similar levels of catalase activity.

Example 5

Identification of the InhA Gene of M. smegmatis

A spontaneous INH-ETH-resistant mutant of M. smegmatis, mc^2651, was isolated from wt M. smegmatis in a single step with a mutational frequency of 10^{-7}. A genomic library from mc^2651 was constructed in a multicopy (5 to 10 copies) shuttle cosmid vector; the vector was described by Y. Zhang et al., Mol. Microbiol. 8, 521 (1993). Upon transfer of the library into wt M. smegmatitis strains,

cosmid clones were identified that consistently conferred INH-ETH resistance. These results are shown in the table in Figure 1.

Cells of M. smegmatis mc^2155 bearing the indicated plasmids (derived from insertion into pYUB18) were grown in 7H9 broth containing kanamycin (15μg/ml), and dilutions were plated on 7H10 agar plates containing kanamycin alone or kanamycin with various concentrations of INH or ETH. The strains without any plasmid were grown in 7H9 broth, and dilutions were plated on 7H10 agar plates and on 7H10 agar plates with various concentrations of INH or ETH.

The transformation of cosmids containing a cross-hybridizing DNA fragment from wt (INH-ETH-sensitive strains) of M. smegmatis, M. tuberculosis, M. bovis, M. bovis BCG, and M. avium yielded clones that conferred INH-ETH resistance. The INH-ETH resistance conferred by the transfer of the wt DNA fragment could be due to overexpression of the target, as is the case for the resistance phenotype seen with several antibiotics.

A 3-kb Bam HI DNA fragment from the M. smegmatis cosmid pYUB286 that conferred INH resistance was used as a probe for Southern (DNA) analysis. This probe strongly hybridized to all of the 11 different mycobacterial species tested, including the pathogenic strains M. tuberculosis, M. bovis, M. avium, and M. leprae, demonstrating that this sequence is highly conserved among the mycobacteria.

The DNA fragments hybridizable with those that conferred resistance to INH were isolated from the wt (INH-sensitive) strains of M. smegmatis, M. bovis, and M. tuberculosis, as well as from the INH-resistant mutants of M. smegmatis and M. bovis. Sequence analysis revealed that each strain contains two open reading frames (ORFs), one encoding a 29-kD protein followed by another encoding a 32-kD protein. Figure 2 presents the DNA sequences of INH-

resistant polynucleotides from M. tuberculosis, M. bovis, and M. smegmatis. The point mutation that differs between the INH-resistant and INH-sensitive M. smegmatis strains, and that determines resistance, is shown in the Figure.

Subcloning studies to determine the smallest fragments to confer INH-resistance were performed; the strategy is shown in Figure 3. In the Figure, panel A is subcloning of M. smegmatis mc^2155, and panel B is of M. tuberculosis H37Rv. The M. smegmatis mc^2155 were transformed with a pool of E. coli-mycobacteria shuttle cosmids, and individual clones were scored for resistance (r,+) or sensitivity (-) to INH and ETH. The ORF preceding inhA is labeled orf1 and the sequence of the intervening DNA is shown. The ribosome binding sites are indicated in boldface letters. The following enzymes were used for subcloning: B, Bam HI; P, Pst I;, S, Spe I, V, Pvu II, N, Nla III; G, Bgl II, H, Nhe I. All the subclones were tested in both orientations. Subcloning analysis of M. bovis DNA yielded results similar to those obtained with M. tuberculosis. Plasmid pYUB291 was also shown to confer INH and ETH resistance in M. bovis BCG host.

The subcloning studies demonstrated that the second ORF from M. smegmatis was sufficient to confer the INH-resistance phenotype. This second ORF was thus named the inhA gene. In contrast to the M. smegmatis gene, the M. tuberculosis and M. bovis inhA genes appear to be in an operon with the gene encoding the 29-kD ORF, an observation confirmed by subcloning. In M. tuberculosis and M. bovis DNA, the noncoding region between the two ORFs was substantially shorter than that in M. smegmatis and may lack a promoter that appears to be present in the latter strain. The inhA DNA sequences have been submitted to GenBank. The accession numbers are U02530 (for M. smegmatis) and U02492 (for M. tuberculosis). The M. bovis sequence is identical to that of M. tuberculosis.

The InhA protein may use nicotinamide or flavin nucleotides as substrates or cofactors, as translation of the putative protein encoded therein indicates that it has a putative binding site for these molecules.

Example 6

Effect of InhA on Mycolic Acid Biosynthesis

As shown in Figure 4, the predicted InhA proteins of M. tuberculosis, M. bovis, and M. smegmatis show strong sequence similarity (about 40% identity over 203 amino acids) to the EnvM proteins of S. typhimurium and E. coli. The figure aligns the amino acid sequences of InhA proteins from the indicated strains with the EnvM proteins from E. coli and S. typhimurium. The amino acid sequences were obtained by conceptual translation of the inhA and envM ORFs. Over a stretch of 203 amino acids, InhA and EnvM show about 75% sequence similarity (40% identity). InhA is highly conserved among mycobacterial strains. The InhA proteins of M. tuberculosis H37Rv and M. bovis are identical and hence are represented by a single sequence. The M. tuberculosis-M. bovis InhA has greater than 95% identity with the M. smegmatis InhA. The various envM gene products are also highly conserved (98% identity)(F. Turnowsky et al., J. Bacteriol. 171, 6555 (1989); H. Bergler et al., J. Gen. Microbiol. 138, 2093 (1992). The protein EnvM is thought to be involved in fatty acid biosynthesis. The relatively close homologies suggest that inhA may be involved in lipid biosynthesis.

The effect of inhA on mycolic acid biosynthesis was determined in cell-free assays. The M. smegmatis mc^2155 gene was transformed with pYUB18 vector (strain mc^2144) or pYUB18 carrying the inhA genes of M. smegmatis (pYUB291, product of subcloning of pYUB286, strain mc^2801), M. avium (pYUB317, strain mc^2832), or M. bovis BCG (pYUB316, strain mc^2799). Cell-free extracts were prepared

from each of these strains and from the spontaneous INH-resistant mutant (mc^2651 of M. smegmatis. Incorporation of [1-^{14}C]acetate into mycolic acids was measured using an assay described in L.M. Lopez-Marin et al., Biochim. Biophys. Acta 1086, 22 (1991), after preincubation with or without Inh. Protein concentrations in cell-free extracts were adjusted to 0.37 to 0.50 mg/ml, which resulted in the linear incorporation of radioactivity into the mycolic acids after a 1-hour incubation of the cell-free extract with the radioactive acetate. Each assay was done in duplicate, and the experimental error between different experiments was no more than 15%. The results of the cell-free assays of mycolic acid biosynthesis are shown in Figure 5. The INH concentration necessary for strong inhibition of mycolic acid biosynthesis in cell-free extracts of the sensitive strain was about 20 times greater than the MIC (here, 20 x MIC = 100 μg/ml, solid bars.) Open bars, 0μg/ml; crosshatched bars, 250 μg/ml. A 20- to 50- fold accumulation of INH has been reported to occur inside the mycobacteria.

As seen from the results in Figure 5, compared to wt extracts, cell-free extracts from the resistant mutant mc^2651 or from resistant merodiploids containing multiple copies of inhA showed marked resistance to the INH-mediated inhibition of mycolic acid biosynthesis. This result is supportive of the suggestion that InhA is required for mycolic acid biosynthesis.

Example 7

Allele Exchange of inhA Genes Conferring Inh-Resistance and Sensitivity Phenotypes

The InhA protein from the INH-resistant mutant (mc^2651) differs from the wt (mc^2155) by a single substitution of Ser to Ala at position 94. To test whether this difference caused the INH resistance phenotype in

mc^2651, an allele exchange was performed on the M. smegmatis chromosome. The mc^2651 cells were transformed with a 45 kb M. smegmatis DNA fragment that contained the wt inhA gene linked to a kan^r marker gene.

A 45 kb long DNA fragment containing the inhA allele from mc^2155 was cloned into a vector with Pac I sites flanking the insert, and a Tn5sequ1 transposon (containing the kan^r gene) was introduced near inhA. The linear Pac I fragment containing inhA linked to kan^r was transformed into mc^2651 by electroporation. The transformants were plated on 7H10 plates containing kanamycin (15 mg/ml). The kanamycin-resistant transformants were then scored for INH sensitivity on 7H10 plates containing both kanamycin (15μg/ml) and INH (10μg/ml). INH sensitivity contransformed with kanamycin resistance in 93 of 130 (72%) transformants tested. The remaining transformants were as resistant to INH as was mc^2651 (MIC = 50 μg/ml). Figure 6 presents a diagram of the allelic exchange experiment.

Allelic exchange was confirmed by restriction fragment length polymorphism analysis of the inhA polymerase chain reaction (PCR) products obtained from the recombinants and by Southern blots.

This result provides evidence that the mutation of Ser to Ala^{94} mediates the INH-resistance phenotype in M. smegmatis.

An allelic exchange could not be performed in M. bovis because a homologous recombination system is lacking. However, the mutant M. bovis gene conferred a higher level of resistance to INH (100% survival in 20 μg/ml of INH, MIC = 30 μg/ml) than did the wt M. bovis gene (0% survival in 20 μg/ml of INH, MIC = 15 μg/ml) when transformed into M. smegmatis mc^2155 on a pYUB18 cosmid vector. These results shown in the table in Figure 1, demonstrate that the identical mutation of Ser to Ala caused INH resistance in

M. bovis NZ.

Example 8

Susceptibility of M. tuberculosis in a Clinical Sample to INH: Single Strand Polymorphism Conformation Analysis

A polynucleotide encoding InhA can be used to assess the susceptibility of various strains of M. tuberculosis in a clinical sample to INH.

The chromosomal DNA of M. tuberculosis is isolated from a clinical sample. Oligonucleotides are prepared using the wild-type inhA sequence of M. tuberculosis. This sequence is depicted in Figure 8. Regions of the inhA gene of M. tuberculosis from the clinical sample which are identified by use of the oligonucleotides are amplified using polymerase chain reaction (PCR) to obtain double stranded DNA. Next, in order to determine whether a mutant inhA gene exists, single strand conformation polymorphism analysis is performed. An example of single strand conformation polymorphism analysis is described by Telenti et al. in "Detection of Rifampicin-Resistance Mutations in Mycobacterium Tuberculosis", Vol. 341 pages 647-650 (March 1993).

In order to perform single strand conformation polymorphism, PCR is performed after substitution of half of the dCTP by ^{32}P-α-dCTP or chemiluminescent substrates per reaction to generate a labelled 157 bp product. After amplification, the PCR product is diluted to an appropriate concentration with dilution buffer. An aliquot of diluted product is mixed with an appropriate aliquot of sequence loading buffer (Sequenase kit), heated for ten minutes at about 94°C, cooled on ice and loaded onto a non-denaturing sequencing format 0.5% MDE gel (Hydrolink, AT Biochem, Malvern, Penn.) Electrophoresis is then performed at room

termperature and constant power overnight. The gels are then dried and exposed for autoradiography overnight.

Claims

1. An isolated wild-type gene which encodes an enzyme which is the target of action for isoniazid.

2. An isolated wild-type gene which encodes a polypeptide (InhA) which is the target of action for isoniazid (INH).

3. A wild-type gene according to claim 2, wherein the gene is selected from the group consisting of that in M. tuberculosis, M. avium, M. smegmatis, and M. bovis.

4. An isolated mutant gene that encodes InhA wherein the mutant gene is associated with INH-resistance.

5. An isolated polynucleotide encoding an InhA polypeptide or fragment or variant thereof.

6. A polynucleotide according to claim 5, wherein the polynucleotide is a recombinant expression vector comprised of control sequences operably linked to a segment encoding the InhA polypeptide of fragment or variant thereof.

7. A host cell comprised of a polynucleotide selected from the group of polynucleotides according to claim 2, or claim 3, or claim 4, or claim 5, or claim 6.

8. A method of treating an individual for infection caused by a member of the mycobacterial complex comprising:

(a) providing a composition comprised of a polynucleotide capable of inhibiting mRNA activity from an

inhA operon of the infecting species and a suitable excipient; and

(b) administering a pharmacologically effective amount of said composition to the individual.

9. The method of claim 6 wherein the mode of administration of the polynucleotides is selected from oral, enteral, subcutaneous, intraperitoneal and intravenous.

10. A method of assessing susceptibility of a strain of mycobacteria in a biological sample to INH comprising:

(a) providing the mycobacterial DNA from the biological sample;

(b) amplifying a region of the inhA operon;

(c) determining whether a mutation exists within the inhA operon from the biological sample, the presence of the mutation indicating that said mycobacterial strain is resistant to INH.

11. The method of claim 10 wherein the amplification is by a polymerase chain reaction (PCR).

12. The method of claim 11 further comprised of providing a comparable portion of wild-type INH-sensitive inhA operon from the mycobacteria, and the determination of whether a mutation exists in the biological sample is by comparison with the wild-type inhA operon.

13. The method of claim 12, wherein determining whether a mutation exists is performed by single strand conformation polymorphism analysis.

14. A method of determining whether a drug is

effective against mycobacterial infection comprising:

(a) providing isolated InhA;

(b) providing a candidate drug;

(c) mixing InhA with substrates for mycolic acid biosynthesis in the presence or absence of the candidate drug; and

(d) measuring the inhibition of biosynthesis of mycolic acid caused by the presence of the drug, if any.

15. A method of producing a tuberculosis-specific mycolic acid comprising adding purified InhA to substrates required for the biosynthesis of mycolic acid.

16. A method for producing a compound that inhibits InhA activity comprising:

a. providing purified InhA;

b. determining the molecular structure of said InhA;

c. creating a compound with a similar molecular structure to INH; and

d. determining that said compound inhibits the biochemical activity of InhA.

17. An isolated InhA polypeptide or fragment or variant thereof.

18. A recombinant mycobacterial vaccine comprised of attenuated mutants selected from the group consisting of BCG, M. tuberculosis, and M. bovis, wherein the mutants are host cells containing a mutated inhA gene.

FIGURE 1

PLASMID	DESCRIPTION	SOURCE OF INSERT	MIC (µg/ml)	
			INH	ETH
pYUB18	Vector	-	5	20
pYUB314	pYUB18 :: *inhA*	*M. smegmatis*, mc^2155	60	>80
pYUB286	pYUB18 :: *inhA*	*M. smegmatis*, mc^2651	60	>80
pYUB315	pYUB18 :: *inhA*	*M. tuberculosis*	15	>30
pYUB316	pYUB18 :: *inhA*	*M. bovis* BCG	15	>30
pYUB370	pYUB18 :: *inhA*	*M. bovis*	20	>30
pYUB317	pYUB18 :: *inhA*	*M. avium*	60	>80

FIGURE 2

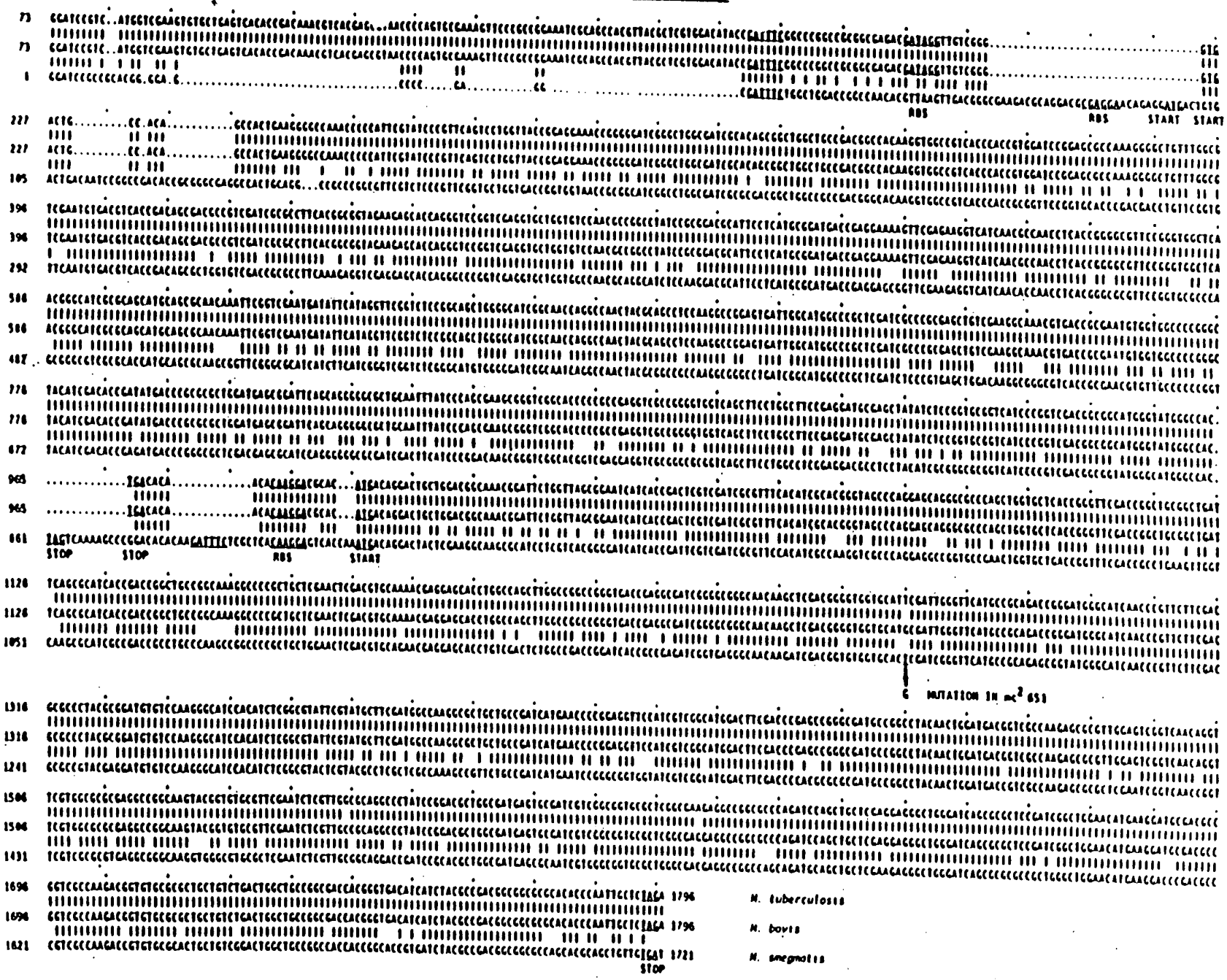
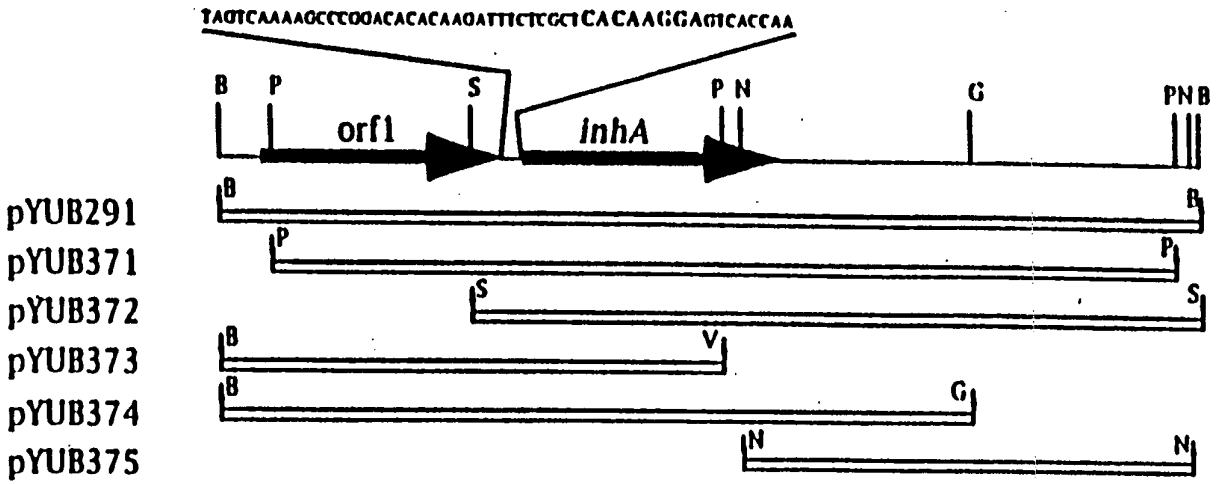
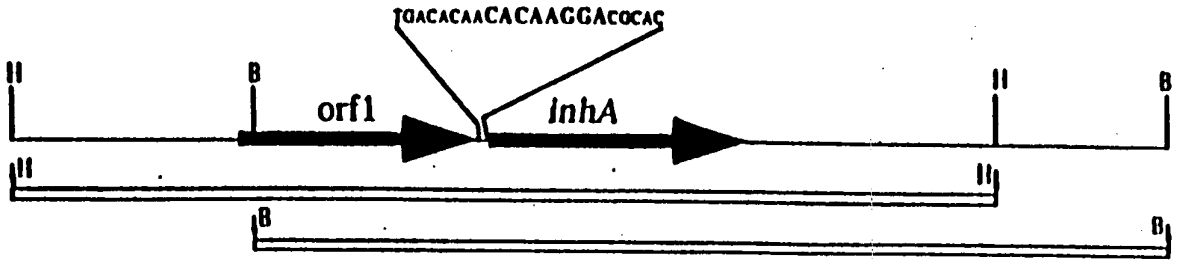

RBS
START
START
RBS
STOP
STOP
RBS
START
G MUTATION IN mc² 651
STOP
M. tuberculosis
M. bovis
M. smegmatis

FIGURE 3





PLASMIDS	*M. smegmatis*, mc²155 INH^r	*M. smegmatis*, mc²155 ETH^r	*M. bovis*, BCG INH^r
pYUB18	-	-	-
pYUB291	+	+	+
pYUB371	+	+	ND
pYUB372	+	+	ND
pYUB373	-	-	ND
pYUB374	+	+	ND
pYUB375	-	-	ND
pYUB376	+	+	ND
pYUB377	-	-	ND

B=*BamHI*, P=*PstI*, S= *SpeI*, V=*PvuII*, G= *BglII*, N=*NlaIII*, H=*NheI*

FIGURE 4

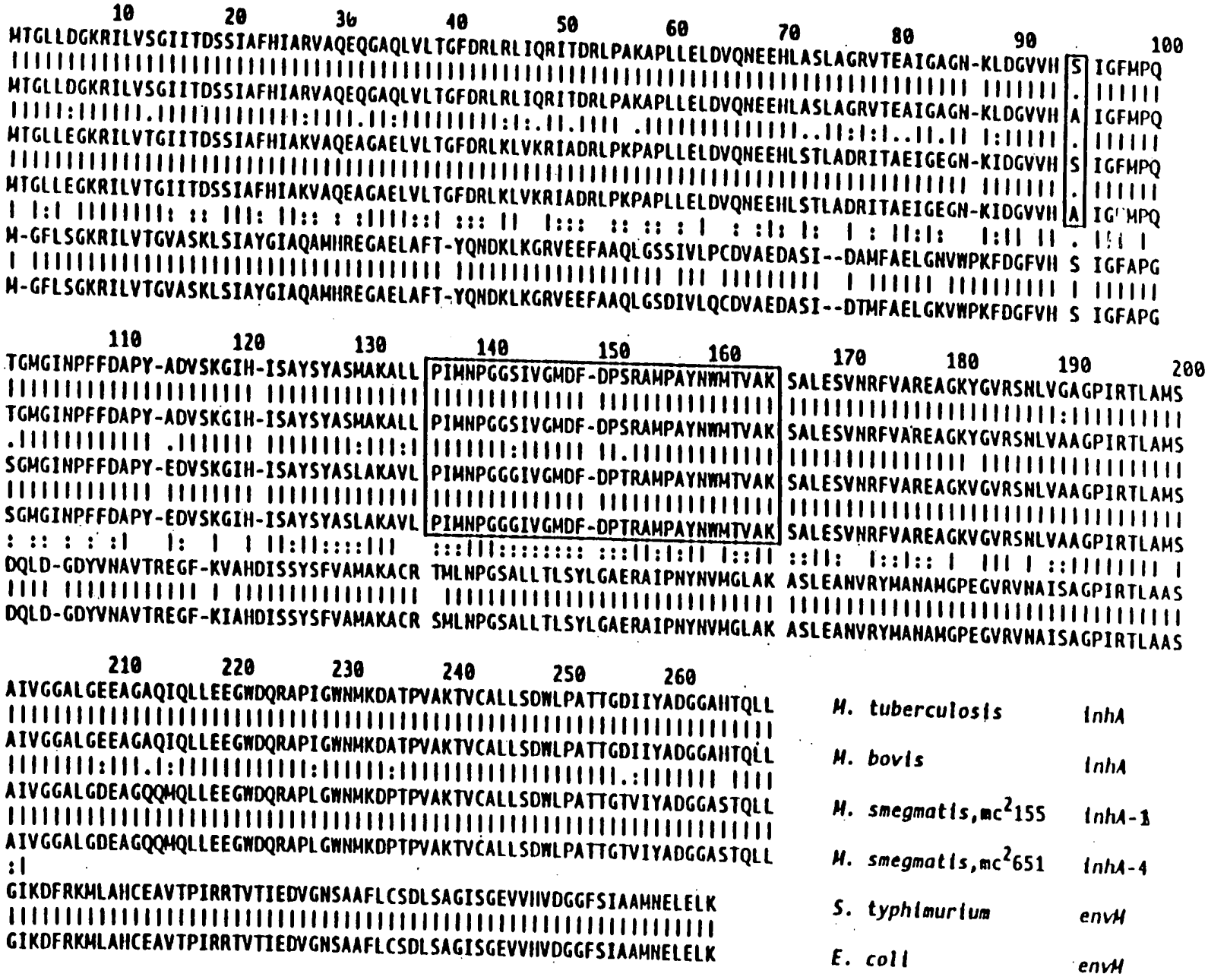
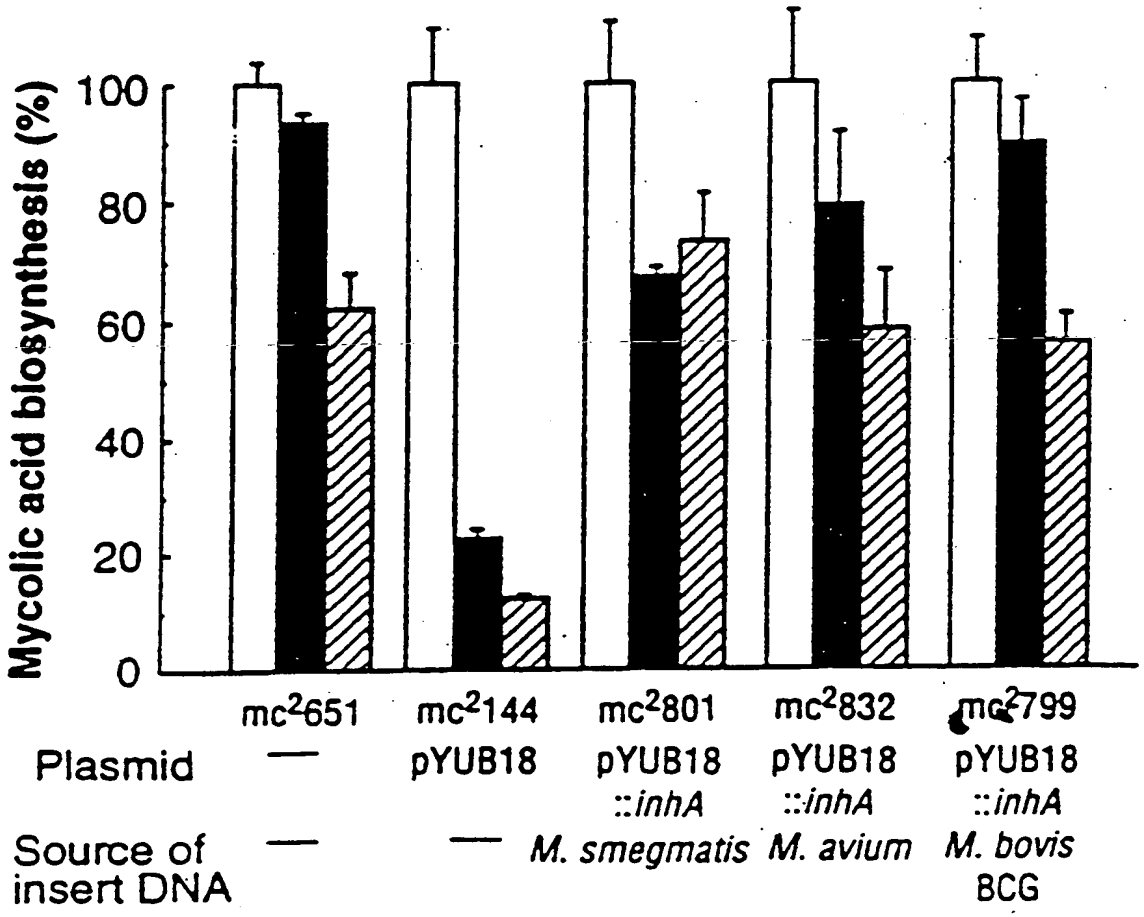
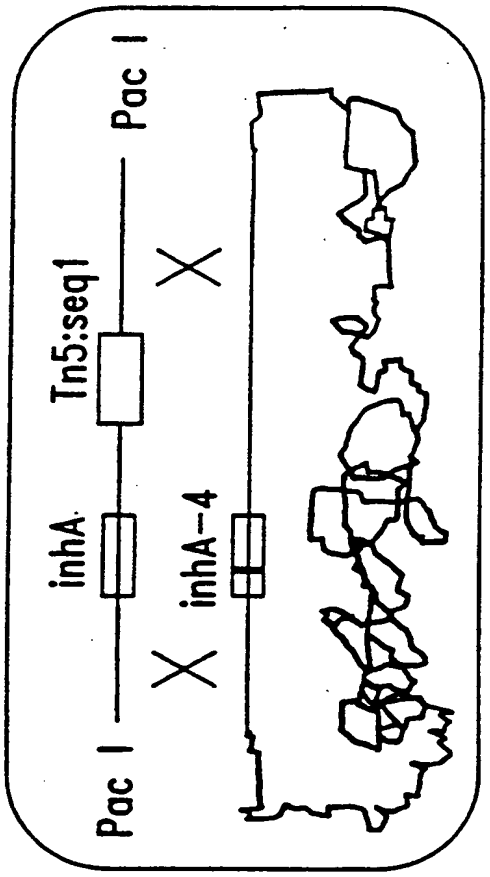

M. tuberculosis inhA
M. bovis inhA
M. smegmatis,mc²155 inhA-1
M. smegmatis,mc²651 inhA-4
S. typhimurium envM
E. coli envM

FIGURE 5


Mycolic acid biosynthesis (%)
100
80
60
40
20
0
mc²651
mc²144
mc²801
mc²832
mc²799
Plasmid
—
pYUB18
pYUB18 ::inhA
pYUB18 ::inhA
pYUB18 ::inhA
Source of insert DNA
—
—
M. smegmatis
M. avium
M. bovis BCG



mc^2651

No. Transformants		Frequency of Cotransformation of	
Kan^r	Kan^rInh^s	Kan^r	with INH^s
28	19	0.68	

FIG. 6

FIG. 7A-1

SEQUENCE OF M. SMEGMATIS inhA GENE

1	GGATCCGCCG	CACGGGGAGC	CCCGAGGCGA	TTTCTGGCTG	GACCGGCCAA	CACGTTAAGT
	CCTAGGCGGC	GTGCCCCTCG	GGGCTCCGCT	AAAGACCGAC	CTGGCCGGTT	GTGCAATTCA
61	TGACGGGCGA	AGACGCAGGA	CGCGAGGAAC	AGAGGATGAC	TGTGACTGAC	AATCCGGCCG
	ACTGCCCGCT	TCTGCGTCCT	GCGCTCCTTG	TCTCCTACTG	ACACTGACTG	TTAGGCCGGC
121	ACACCGCGGG	CGAGGCCACT	GCAGGCCGCC	CGGCGTTCGT	CTCCCGTTCG	GTGCTGGTGA
	TGTGGCGCCC	GCTCCGGTGA	CGTCCGGCGG	GCCGCAAGCA	GAGGGCAAGC	CACGACCACT
181	CCGGTGGTAA	CCGCGGCATC	GGCCTGGCGA	TCGCGCGACG	GCTGGCCGCC	GACGGGCACA
	GGCCACCATT	GGCGCCGTAG	CCGGACCGCT	AGCGCGCTGC	CGACCGGCGG	CTGCCCGTGT
241	AGGTGGCCGT	CACCCACCGC	GGTTCCGGTG	CACCCGACGA	CCTGTTCGGT	GTTCAATGTG
	TCCACCGGCA	GTGGGTGGCG	CCAAGGCCAC	GTGGGCTGCT	GGACAAGCCA	CAAGTTACAC
301	ACGTCACCGA	CAGCGCTGGT	GTCGACCGCG	CCTTCAAAGA	GGTCGAGGAG	CACCAGGGCC
	TGCAGTGGCT	GTCGCGACCA	CAGCTGGCGC	GGAAGTTTCT	CCAGCTCCTC	GTGGTCCCGG
361	CGGTCGAGGT	GCTGGTGGCC	AACGCAGGCA	TCTCCAAGGA	CGCATTCCTC	ATGCGCATGA
	GCCAGCTCCA	CGACCACCGG	TTGCGTCCGT	AGAGGTTCCT	GCGTAAGGAG	TACGCGTACT
421	CCGAGGAGCG	GTTCGAAGAG	GTCATCAACA	CCAACCTCAC	GGGCGCGTTC	CGGTGCGCCC
	GGCTCCTCGC	CAAGCTTCTC	CAGTAGTTGT	GGTTGGAGTG	CCCGCGCAAG	GCCACGCGGG

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481  AGCGGGCGTC GCGCACCATG CAGCGCAAGC GGTTCGGGCG CATCATCTTC ATCGGGTCGG
     TCGCCCGCAG CGCGTGGTAC GTCGCGTTCG CCAAGCCCGC GTAGTAGAAG TAGCCCAGCC

541  TCTCGGGCAT GTGGGGGATC GGCAATCAGG CCAACTACGC GGCCGCCAAG GCGGGCCTGA
     AGAGCCCGTA CACCCCCTAG CCGTTAGTCC GGTTGATGCG CCGGCGGTTC CGCCCGGACT

601  TCGGCATGGC CCGCTCGATC TCCCGTGAGC TGGACAAGGC GGGCGTCACC GCGAACGTGT
     AGCCGTACCG GGCGAGCTAG AGGGCACTCG ACCTGTTCCG CCCGCAGTGG CGCTTGCACA

661  TGCCCCCCGG TTACATCGAC ACCGAGATGA CCCGGGCGCT CGACGAGCGC ATCCAGGGGG
     ACGGGGGGCC AATGTAGCTG TGGCTCTACT GGGCCCGCGA GCTGCTCGCG TAGGTCCCCC

721  GCGCGATCGA CTTCATCCCG GACAAGCGGG TCGGCACGGT CGAGGAGGTC GCGGGCGCGG
     CGCGCTAGCT GAAGTAGGGC CTGTTCGCCC AGCCGTGCCA GCTCCTCCAG CGCCCGCGCC

781  TCAGCTTCCT GGCCTCGGAG GACGCCTCCT ACATCGCGGG CGCGGTCATC CCCGTCGACG
     AGTCGAAGGA CCGGAGCCTC CTGCGGAGGA TGTAGCGCCC GCGCCAGTAG GGGCAGCTGC

841  GCGGTATGGG CATGGGCCAC TAGTCAAAAG CCCGGACACA CAAGATTTCT CGCTCACAAG
     CGCCATACCC GTACCCGGTG ATCAGTTTTC GGGCCTGTGT GTTCTAAAGA GCGAGTGTTC

901  GAGTCACCAA ATGACAGGCC TACTCGAAGG CAAGCGCATC CTCGTCACGG GGATCATCAC
     CTCAGTGGTT TACTGTCCTG ATGAGCTTCC GTTCGCGTAG GAGCAGTGCC CCTAGTAGTG

961  CGATTCGTCG ATCGCGTTCC ACATCGCCAA GGTCGCCCAG GAGGCCGGCG CCGAACTGGT
     GCTAAGCAGC TAGCGCAAGG TGTAGCGGTT CCAGCGGGTC CTCCGGCCAC GGCTTGACCA

1021 GCTGACCGGT TTCGACCGCC TGAAGTTGGT CAAGCGCATC GCCGACCGCC TGCCCAAGCC
     CGACTGGCCA AAGCTGGCGG ACTTCAACCA GTTCGCGTAG CGGCTGGCGG ACGGGTTCGG
```

FIG. 7A-2

FIG. 7B-1

1081	GGCCCCGCTG	CTGGAACTCG	ACGTGCAGAA	CGAGGAGCAC	CTGTCGACTC	TGGCCGACCG
	CCGGGGCGAC	GACCTTGAGC	TGCACGTCTT	GCTCCTCGTG	GACAGCTGAG	ACCGGCTGGC
1141	GATCACCGCC	GAGATCGGTG	AGGGCAACAA	GATCGACGGT	GTGGTGCACG	CGATCGGGTT
	CTAGTGGCGG	CTCTAGCCAC	TCCCGTTGTT	CTAGCTGCCA	CACCACGTGC	GCTAGCCCAA
1201	CATGCCGCAG	AGCGGTATGG	GCATCAACCC	GTTCTTCGAC	GCGCCGTACG	AGGATGTGTC
	GTACGGCGTC	TCGCCATACC	CGTAGTTGGG	CAAGAAGCTG	CGCGGCATGC	TCCTACACAG
1261	CAAGGGCATC	CACATCTCGG	CGTACTCGTA	CGCCTCGCTC	GCCAAAGCCG	TTCTGCCGAT
	GTTCCCGTAG	GTGTAGAGCC	GCATGAGCAT	GCGGAGCGAG	CGGTTTCGGC	AAGACGGCTA
1321	CATGAATCCG	GGCGGCGGCA	TCGTCGGCAT	GGACTTCGAC	CCCACGCGCG	CGATGCCGGC
	GTACTTAGGC	CCGCCACCAT	AGCAGCCATA	CCTGAAGCTG	GGGTGCGCGC	GCTACGGCCG
1381	CTACAACTGG	ATGACCGTCG	CCAAGAGCGC	GCTCGAATCG	GTCAACCGGT	TCGTCGCGCG
	GATGTTGACC	TACTGGCAGC	GGTTCTCTCG	CGAGCTTAGC	CAGTTGGCCA	AGCAGCGCGC
1441	TGAGGCGGGC	AAGGTGGGCG	TGCGCTCGAA	TCTCGTTGCG	GCAGGACCGA	TCCGCACGCT
	ACTCCGCCCG	TTCCACCCGC	ACGCGAGCTT	AGAGCAACGC	CGTCCTGGCT	AGGCGTGCGA

FIG. 7B-2

1501	GGCGATGAGC	GCAATCGTGG	GCGGTGCGCT	GGGCGACGAG	GCCGGCCAGC	AGATGCAGCT
	CCGCTACTCG	CGTTAGCACC	CGCCACGCGA	CCCGCTGCTC	CGGCCGGTCG	TCTACGTCGA
1561	GCTCGAAGAG	GGCTGGGATC	AGCGCGCGCC	GCTGGGCTGG	AACATGAAGG	ACCCGACGCC
	CGAGCTTCTC	CCGACCCTAG	TCGCGCGCGG	CGACCCGACC	TTGTACTTCC	TGGGCTGCGG
1621	CGTCGCCAAG	ACCGTGTGCG	CACTGCTGTC	GGACTGGCTG	CCGGCCACCA	CCGGCACCGT
	GCAGCGGTTC	TGGCACACGC	GTGACGACAG	CCTGACCGAC	GGCCGGTGGT	GGCCGTGGCA
1681	GATCTACGCC	GACGGCGGCG	CCAGCACGCA	GCTGTTGTGA	TACCGCCGTG	TCGTATGACG
	CTAGATGCGG	CTGCCGCCGC	GGTCGTGCGT	CGACAACACT	ATGGCGGCAC	AGCATACTGC
1741	CCTTGCTACT	GCTGTCGTTC	GACGGGCCGG	AACTCCCGAG	CAGGTGATGC	CGTTCTTGGA
	GGAACGATGA	CGACAGCAAG	CTGCCCGGCC	TTGAGGGCTC	GTCCACTACG	GCAAGAACCT
1801	GAACTCACCA	GGGGCCGCGG	AATCCCCAGG	GAGCGGCTGG	AATCGGTGGC	CGAGCACTAT
	CTTGAGTGGT	CCCCGGCGCC	TTAGGGGTCC	CTCGCCGACC	TTAGCCACCG	GCTCTGGATA
1861	CTGCACTTCG	GCGGGGTGTC	ACCGATCAAC	GGCATCAACC	GGGACCTGAT	CGTCGCGATC
	GACGTGAAGC	CGCCCCACAG	TGGCTAGTTG	CGTAGTTGG	CCCTGGACTA	GCAGCGCTAG
1921	GAGGCCGAAC	TCGCCCGACG	CGGCCGCAAC	CTTCCGGTCT	ACTTCGGCAA	CCGCAACTGG
	CTCCGGCTTG	AGCGGGCTGC	GCCGGCGTTG	GAAGGCCAGA	TGAAGCCGTT	GGCGTTGACC
1981	GAGCCGTACG	TCGAAGACAC	TGTCAAGGCG	ATGTCCGACA	ACGGAATCCG	TCGTGCGGCG
	CTCGGCATGC	AGCTTCTGTG	ACAGTTCCGC	TACAGGCTGT	TGCCTTAGGC	AGCACGCCGC

2041	GTGTTCGCGA	CCTCGGCGTG	GGGTGGGTAC	TCGGGATGCG	CCCAGTACCA	GGAGGACATC
	CACAAGCGCT	GGAGCCGCAC	CCCACCCATG	AGCCCTACGC	GGGTCATGGT	CCTCCTGTAG
2101	GCGCGTGGCC	GGGCCGCCGC	CGGGCCCGAG	GCGCCGGAGC	TGGTCAAGCT	GCGCCAGTAT
	CGCGCACCGG	CCCGGCGGCG	GCCCGGGCTC	CGCGGCCTCG	ACCAGTTCGA	CGCGGTCATA
2161	TTCGACCACC	CGCTGTTCGT	CGAGATGTTC	GCCGACGCCG	TCGCCGACGC	CGCGGCCACC
	AAGCTGGTGG	GCGACAAGCA	GCTCTACAAG	CGGCTGCGGC	AGCGGCTGCG	GCGCCGGTGG
2221	CTGCCCGAGG	AACTGCGGGA	CGAAGCGCGG	CTGGTGTTCA	CCGCCCACTC	CATCCCGCTG
	GACGGGCTCC	TTGACGCCCT	GCTTCGCGCC	GACCACAAGT	GGCGGGTGAG	GTAGGGCGAC

FIG. 7B-3

FIG. 7C-1

2281	CGTGCCGCGT	CGCGTTGCGG	TGCAGATCTC	TACGAGCGGC	AGGTGGGTTA	CGCCGCGCGG
	GCACGGCGCA	GCGCAACGCC	ACGTCTAGAG	ATGCTCGCCG	TCCACCCAAT	GCGGCGCGCC
2341	CTGGTCGCGG	CCGCAGCCGG	GTACCGCGAA	TACGACCAGG	TATGGCAGTC	CCGGTCCGGC
	GACCAGCGCC	GGCGTCGGCC	CATGGCGCTT	ATGCTGGTCC	ATACCGTCAG	GGCCAGGCCG
2401	CCGCCGCAGG	TGCCGTGGCT	CGAACCCGAC	GTCGGAGATC	ACCTTGAGGC	GTTGGCGCGC
	GGCGGCGTCC	ACGGCACCGA	GCTTGGGCTG	CAGCCTCTAG	TGGAACTCCG	CAACCGCGCG
2461	AACGGCACCA	GGGCGGTCAT	CGTGTGTCCC	CTCGGCTTCG	TCGCCGACCA	CATCGAGGTG
	TTGCCGTGGT	CCCGCCAGTA	GCACACAGGG	GAGCCGAAGC	AGCGGCTGGT	GTAGCTCCAC
2521	GTGTGGGATC	TGGACAACGA	ACTGGCCGAG	CAGGCCGCCG	AGGCAGGCAT	CGCGTTCGCG
	CACACCCTAG	ACCTGTTGCT	TGACCGGCTC	GTCCGGCGGC	TCCGTCCGTA	GCGCAAGCGC
2581	CGTGCCGCCA	CGCCCAACTC	CCAGCCACGT	TTTGCCCAAC	TTGTCGTCGA	CCTGATCGAC
	GCACGGCGGT	GCGGGTTGAG	GGTCGGTGCA	AAACGGGTTG	AACAGCAGCT	GGACTAGCTG
2641	GAAATGCTGC	ACGGACTTCC	GCCACGCCGG	GTCGAGGGGC	CCGATCCGTG	CCCGCCTACG
	CTTTACGACG	TGCCTGAAGG	CGGTGCGGCC	CAGCTCCCCG	GGCTAGGCAC	GGGCGGATGC
2701	GCAGCAGTGT	CAACGGCGCA	CCGTGCACGC	CGGCCTGCTC	GGCGTGACCC	GCCCCGGGCG
	CGTCGTCACA	GTTGCCGCGT	GGCACGTGCG	GCCGGACGAG	CCGCACTGGG	CGGGGCCCGC

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2761  CAGCGAGTCG  GGCCGGGCGA  GCAAGAACGC  CAGGCGGAAT  GCAGGATCGC  CTCGAGTGCG
      GTCGCTCAGC  CCGGCCCGCT  AGTTCTTGCG  GTCCGCCTTA  CGTCCTAGCG  GAGCTCACGC

2821  GCCATACGCG  CCGAGCGCAC  CACCCGCGTG  AGGGGGCGCA  GCGCCGAGTC  GGCGATCTGA
      CGGTATGCGC  GGCTCGCGTG  GTGGGCGCAC  TCCCCCGCGT  CGCGGCTCAG  CCGCTAGACT

2881  ACCTCCGACG  AACTCTGCAG  ACCGCTCGGG  ATCAGACCCG  CACTCACCGC  GATGATGGCG
      TGGAGGCTGC  TTGAGACGTC  TGGCGAGCCC  TAGTCTGGGC  GTGAGTGGCG  CTACTACCGC

2941  TCGACATGGG  CGGCGTTCTC  CAGCACCCGC  ACAGCCCGGG  TCGGCGCGTG  GTCGGGGACG
      AGCTGTACCC  GCCGCAAGAG  GTCGTGGGCG  TGTCGGGCCC  AGCCGCGCAC  CAGCCCCTGC

3001  CGGTGCGCGC  GCCCGGCGGC  GAGGATCTGC  TCGACCATCC  CGCGCGGATC  C
      GCCACGCGCG  CGGGCCGCCG  CTCCTAGACG  AGCTGGTAGG  GCGCGCCTAG  G
```

FIG. 7C-2

FIG. 8A-1

SEQUENCE OF THE M. TUBERCULOSIS inhA GENE

1	AGCGCGACAT	ACCTGCTGCG	CAATTCGTAG	GGCGTCAATA	CACCCGCAGC	CAGGGCCTCG
	TCGCGCTGTA	TGGACGACGC	GTTAAGCATC	CCGCAGTTAT	GTGGGCGTCG	GTCCCGGAGC
61	CTGCCCAGAA	AGGGATCCGT	CATGGTCGAA	GTGTGCTGAG	TCACACCGAC	AAACGTCACG
	GACGGGTCTT	TCCCTAGGCA	GTACCAGCTT	CACACGACTC	AGTGTGGCTG	TTTGCAGTGC
121	AGCGTAACCC	CAGTGCGAAA	GTTCCCGCCG	GAAATCGCAG	CCACGTTACG	CTCGTGGACA
	TGTGGCGCCC	GCTCCGGTGA	CGTCCGGCGG	CGGCGTTCGT	GAGGGCAAGC	CACGACCACT
181	TCGCATTGGG	GGCCCGGCCG	CGGCGAGACG	ATAGGTTGTC	GGGGTGACTG	CCACAGCCAC
	ATGGCTAAAG	CCGGGCCGGC	CCCGCTCTGC	TATCCAACAG	CCCCACTGAC	GGTGTCGGTG
241	TGAAGGGGCC	AAACCCCCAT	TCGTATCCCG	TTCAGTCCTG	GTTACCGGAG	GAAACCGGGG
	ACTTCCCCGG	TTTGGGGGTA	AGCATAGGGC	AAGTCAGGAC	CAATGGCCTC	CTTTGGCCCC
301	GATCGGGCTG	GCGATCGCAC	AGCGGCTGGC	TGCCGACGGC	CACAAGGTGG	CCGTCACCCA
	CTAGCCCGAC	CGCTAGCGTG	TCGCCGACCG	ACGGCTGCCG	GTGTTCCACC	GGCAGTGGGT
361	CCGTGGATCC	GGAGCGCCAA	AGGGGCTGTT	TGGCCTCGAA	TGTGACGTCA	CCGACAGCGA
	GGCACCTAGG	CCTCGCGGTT	TCCCCGACAA	ACCGCAGCTT	ACACTGCAGT	GGCTGTCGCT
421	CGCCGTCGAT	CGCGCCTTCA	CGGCGGTAGA	AGAGCACCAG	GGTCCGGTCG	AGGTGCTGGT
	GCGGCAGCTA	GCGCGGAAGT	GCCGCCATCT	TCTCGTGGTC	CCAGGCCAGC	TCCACGACCA

FIG. 8A-2

```
481   GTCCAACGCC GGCCTATCCG CGGACGCATT CCTCATGCGG ATGACCGAGG AAAAGTTCGA
      CAGGTTGCGG CCGGATAGGC GCCTGCGTAA GGAGTACGCC TACTGGCTCC TTTTCAAGCT

541   GAAGGTCATC AACGCCAACC TCACCGGGGC GTTCCGGGTG ATGACCGAGG AAAAGTTCGA
      CAGGTTGCGG CCGGATAGGC GCCTGCGTAA GGAGTACGCC TACTGGCTCC TTTTCAAGCT

601   CATGCAGCGC GGCCTATCCG CGGACGCATT CCTCATGCGG ATGACCGAGG AAAAGTTCGA
      CAGGTTGCGG CCGGATAGGC GCCTGCGTAA GGAGTACGCC TACTGGCTCC TTTTCAAGCT

661   CATCGGCAAC CAGGCCAACT ACGCAGCCTC CAAGGCCGGA GTGATTGGCA TGGCCCGCTC
      GTAGCCGTTG GTCCGGTTGA TGCGTCGGAG GTTCCGGCCT CACTAACCGT ACCGGGCGAG

721   GATCGCCCGC GAGCTGTCGA ACGCAGCCTC CAAGGCCGGA GTGATTGGCA CGGGCTACAT
      CTAGCGGGCG CTCGACAGCT TCCGTTTGCA CTGGCGCTTA CACCACCGGG GCCCGATGTA

781   CGACACCGAT ATGACCCGCG CGCTGGATGA GCGGATTCAG GAGGGGCGC TGCAATTTAT
      GCTGTGGCTA TACTGGGCGC GCGACCTACT CGCCTAAGTC GTCCCCCGCG ACGTTAAATA

841   CCCAGCGAAG ATGACCCGCG CGCTGGATGA GCGGATTCAG CAGGGGCGC TCCTGGCTTC
      GGGTCGCTTC GCCCAGCCGT GGGGGCGGCT CCAGCGGCCC CACCAGTCGA AGGACCGAAG

901   CGAGGATGCG AGCTATATCT CCGGTGCGGT CATCCCGGTC GACGGCGGCA TGGGTATGGG
      GCTCCTACGC TCGATATAGA GGCCACGCCA GTAGGGCCAG CTGCCGCCGT ACCCATACCC

961   CCACTGACAC AACACAAGGA CGCACATGAC AGGACTGCTG GACGGCAAAC GGATTCTGGT
      GGTGACTGTG TTGTGTTCCT GCGTGTACTG TCCTGACGAC CTGCCGTTTG CCTAAGACCA

1021  TAGCGGAATC ATCACCGACT CGTCGATCGC GTTTCACATC GCACGGGTAG CCCAGGAGCA
      ATCGCCTTAG TAGTGGCTGA GCAGCTAGCG CAAAGTGTAG CGTGCCCATC GGGTCCTCGT
```

FIG. 8B-1

1081	GGGCGCCCAG	CTGGTGCTCA	CCGGGTTCGA	CCGGCTGCGG	CTGATTCAGC	GCATCACCGA
	CCCGCGGGTC	GACCACGAGT	GGCCCAAGCT	GGCCGACGCC	GACTAAGTCG	CGTAGTGGCT
1141	CCGGCTGCCG	GCAAAGGCCC	CGCTGCTCGA	ACTCGACGTG	CAAAACGAGG	AGCACCTGGC
	GGCCGACGGC	CGTTTCCGGG	GCGACGAGCT	TGACGTGCAC	GTTTTGCTCC	TCGTGGACCG
1201	CAGCTTGGCC	GGCCGGGTGA	CCGAGGCGAT	CGGGGCGGGC	AACAAGCTCG	ACGGGGTGGT
	GTCGAACCGG	CCGGCCCACT	GGCTCCGCTA	GCCCCGCCCG	TTGTTCGAGC	TGCCCCACCA
1261	GCATTCGATT	GGGTTCATGC	CGCAGACCGG	GATGGGCATC	AACCCGTTCT	TCGACGCGCC
	CGTAAGCTAA	CCCAAGTACG	GCGTCTGGCC	CTACCCGTAG	TTGGGCAAGA	AGCTGCGCGG
1321	CTACGCGGAT	GTGTCCAAGG	GCATCCACAT	CTCGGCGTAT	GGCATGGACT	TCGACCCGAG
	GATGCGCCTA	CACAGGTTCC	CGTAGGTGTA	AAGGTAGCAG	CCGTACCTGA	AGCTGGGCTC
1381	GGCGCTGCTG	CCGATCATGA	ACCCCGGAGG	TTCCATCGTC	GGCATGGACT	TCGACCCGAG
	CCGCGACGAC	GGCTAGTACT	TGGGGCCTCC	AAGGTAGCAG	CCGTACCTGA	AGCTGGGCTC
1441	CCGGGCGATG	CCGGCCTACA	ACTGGATGAC	GGTCGCCAAG	AGCGCGTTGG	AGTCGGTCAA
	GGCCCCGCTAC	GGCCCGGATGT	TGACCTACTG	CCAGCGGTTC	TCGCGCAACC	TCAGCCAGTT
1501	CAGGTTCGTG	GCGCGCGAGG	CCGGCAAGTA	CGGTGTGCGT	TCGAATCTCG	TTGGCGCAGG
	GTCCAAGCAC	CGCGCGCTCC	GGCCGTTCAT	GCCACACGCA	AGCTTAGAGC	AACCGCGTCC

FIG. 8B-2

1561	CCCTATCCGG	ACGCTGGCGA	TGAGTGCGAT	CGTCGGCGGT	GCGCTCGGCG	AAGAGGCCGG
	GGGATAGGCC	TGCGACCGCT	ACTCACGCTA	GCAGCCGCCA	CGCGAGCCGC	TTCTCCGGCC
1621	CGCCCAGATC	CAGCTGCTCG	AGGAGGGCTG	GGATCAGCGC	GCTCCGATCG	GCTGGAACAT
	GCGGCTCTAG	GTCGACGAGC	TCCTCCCGAC	CCTAGTCGCG	CGAGGCTAGC	CGACCTTGTA
1681	GAAGGATGCG	ACGCCGGTCG	CCAAGACGGT	GTGCGCGCTG	CTGTCTGACT	GGCTGCCGGC
	CTTCCTACGC	TGCGGCCAGC	GGTTCTGCCA	CACGCGCGAC	GACAGACTGA	CCGACGGCCG
1741	GACCACGGGT	GACATCATCT	ACGCCGACGG	CGGCGCGCAC	ACCCAATTGC	TCTAGAACGC
	CTGGTGCCCA	CTGTAGTAGA	TGCGGCTGCC	GCCGCGCGTG	TGGGTTAACG	AGATCTTGCG
1801	ATGCAATTTG	ATGCCGTCCT	GCTGCTGTCG	TTCGGCCGAC	CGGAAGGGCC	CGAGCAGGTG
	TACGTTAAAC	CTGTAGTAGA	TGCGGCTGCC	GCCGCGCGTG	GCCTTCCCGG	GCTCGTCCAC
1861	CGCCCGTTCC	TGGAGAACGT	TACCCGGGGC	CGCGGTGTGC	CTGCCGAACG	GTTGGACGCG
	GCGGGCAAGG	ACCTCTTGCA	ATGGGCCCCG	GCGCCACACG	GACGGCTTGC	CAACCTGCGC
1921	GTGGCCGAGC	ACTACCTGCA	TTTCGGTGGG	GTATCACCGA	TCAATGGCAT	TAATCGCACA
	CACCGGCTCG	TGATGGACGT	AAAGCCACCC	CATAGTGGCT	AGTTACCGTA	ATTAGCGTGT
1981	CTGATCGCGC	AGCTGGAGGC	GCAGCAAGAA	CTGCCGGTGT	ACTTCGGTAA	CCGCAACTGG
	GACTAGCGCC	TCGACCTCCG	CGTCGTTCTT	GACGGCCACA	TGAAGCCATT	GGCGTTGACC

FIG. 8B-3

```
2041  GAGCCGTATG  TAGAAGATGC  CGTTACGGCC  ATGCGCGACA  ACGGTGTCCG  GCGTGCAGCG
      CTCGGCATAC  ATCTTCTACG  GCAATGCCGG  TACGGCTGT   TGCCACAGGC  CGCACGTCGC

2101  GTCTTTGCGA  CATCTGCGTG  GAGCGGTTAC  TCGAGCTGCA  CACAGTACGT  GGAGGACATC
      CAGAAACGCT  GTAGACGCAC  CTCGCCAATG  AGCTCGACGT  GTGTCATGCA  CCTCCTGTAG

2161  GCGCGGCCCC  CCGCGCGGCC  GGGCGCGACG  CGCCTGAACT  GGTAAAACTG  CGGCCCTACT
      CGCGCCGGGG  GGCGCGCCGG  CCCGCGCTGC  GCGGACTTGA  CCATTTTGAC  GCCGGGATGA

2221  TCGACCATCC  GCTGTTCGTC  GAGATGTTCG  CCGACGCCAT  CACCGCGGCC  GCCGCAACCG
      AGCTGGTAGG  CGACAAGCAG  CTCTACAAGC  GGCTGCGGTA  GTGGCGCCGG  CGGCGTTGGC
```

FIG. 8C-1

```
2281  TGCGCGGTGA TGCCCGGCTG GTGTTCACCG CGCATTCGAT CCCGACGGCC GCCGACCGCC
      ACGCGCCACT ACGGGCCGAC CACAAGTGGC GCGTAAGCTA GGGCTGCCGG CGGCTGGCGG

2341  GCTGTGGCCC CAACCTCTAC AGCCGCCAAG TCGCCTACGC CACAAGGCTG GTCGCGGCCG
      CGACACCGGG GTTGGAGATG TCGGCGGTTC AGCGGATGCG GTGTTCCGAC CAGCGCCGGC

2401  CTGCCGGATA CTGCGACTTT GACCTGGCCT GGCAGTCGAG ATCGGGCCCG CCGCAGGTGC
      GACGGCCTAT GACGCTGAAA CTGGACCGGA CCGTCAGCTC TAGCCCGGGC GGCGTCCACG

2461  GGACCGACCT GCCAGACGTT ACCGACCAGC TCACCGGTCT GGCTGGGCCC GGCATCAACG
      GGACCGACCT CGGTCTGCAA TGGCTGGTCG AGTGGCCAGA CCGACCCCGG CCGTAGTTGC

2521  CGGTGATCGT GTGTCCCATT GGATTCGTCG CCGACCATAT CGAGGTGGTG TGGGATCTCG
      GCCACTAGCA CACAGGGTAA CCTAAGCAGC GGCTGGTATA GCTCCACCAC ACCCTAGAGC

2581  ACCACGAGTT GCGATTACAA GCCGAGGCAG CGGGCATCGC GTACGCCCGG GCCAGCACCC
      TGGTGCTCAA CGCTAATGTT CGGCTCCGTC GCCCGTAGCG CATGCGGGCC CGGTCGTGGG

2641  CCAATGCCGA CCCGCGGTTC GCTCGACTAG CCAGAGGTTT GATCGACGAA CTCCGTTACG
      CGGCATATGG GGGCGCCAAC CGAGCTGATC GGTCTCCAAA CTAGCTGCTT GAGGCAATGC

2701  GCCGTATACC TGCGCGGGTG AGTGGCCCCG ATCCGGTGCC GGGCTGTCTG TCCAGCATCA
      CGGCATATGG ACGCGCCCAC TCACCGGGGC TAGGCCACGG CCCGACAGAC AGGTCGTAGT
```

FIG. 8C-2

```
2761  ACGGCCAGCC ATGCCGTCCG CCGCACTGCG TGGCTAGCGT CAGTCCGGCC AGGCCGAGTG
      TGCCGGTCGG TACGGCAGGC GGCGTGACGC ACCGATCGCA GTCAGGCCGG TCCGGCTCAC

2821  CAGGATCGCC GCACGCTGAG ACATCCGGGC CGAGCGCACC ACGGCGGTCA ACGGTCTCAA
      GTCCTAGCGG CACTGGCGCC TGTAGGCCCG GCTCGCGTGG TGCCGCCAGT TGCCAGAGTT

2881  CGCATCGGTG GCACGCTGAG CGTCCGACAA CGACTGCGTT CCGATCGGCA ATCGACTCAG
      GCGTAGCCAC CGTGCGACTC GCAGGCTGTT GCTGACGCAA GGCTAGCCGT TAGCTGAGTC

2941  CCCGGCACTG ACCGCGATGA TCGCATCGAC GTGCGCGGCA TTCTCGAGCA CCCGCAATGC
      GGGCCGTGAC TGGCGCTACT AGCGTGCTG CACGCGCCGT AAGAGCTCGT GGGCGTTACG

3001  GCGCGATGGC GCGTGGTCGG GAACCCGGTG TTGCCGTGAC GATTCGAGCA ACTGCTCGAC
      CGCGCTACCG CGCACCAGCC CTTGGGCCAC AACGGCACTG CTAAGCTCGT TGACGAGCTG

3061  GAGGCCACGG GGCTTGGCGA CGTCGCTAGA TCCCAGTCCG ATGGTGCTCA AGGCTTCGGC
      CTCCGGTGCC CCGAACCGCT GCAGCGATCT AGGGTCAGGC TACCACGAGT TCCGAAGCCG
```

AMINO ACID SEQUENCE OF PS5

```
      GTTCGCTCCGGCGCGGTCACGCGCATGCCCTCGATGACGCAGATCTCGTCGGGCTCGATG
    1 ---------+---------+---------+---------+---------+---------+
      CGCTCTTCCCAGACTTGCAGCCCCGGGGCACGGCGGCGGTTGGTGTCGATGATCGCGGCG
   61 ---------+---------+---------+---------+---------+---------+
      GGAAGATCCGCGTCGATCCACTTGGCGCCATGGAAGGCAGAAGCCGAGTAGCCGGCCAGC
  121 ---------+---------+---------+---------+---------+---------+
      ACGCCGCGGCGGCGCGAGCGCAGCCACAGCGCTTTTGCACGCAATTGCGCGGTCAGTTCC
  181 ---------+---------+---------+---------+---------+---------+
      ACACCCTGCGGCACGTACACGTCTTTATGTAGCGCGACATACCTGCTGCGCAATTCGTAG
  241 ---------+---------+---------+---------+---------+---------+
      GGCGTCAATACACCCGCAGCCAGGGCCTCGCTGCCCAGAAAGGGATCCGTCATGGTCGAA
  301 ---------+---------+---------+---------+---------+---------+
      GTGTGCTGAGTCACACCGACAAACGTCACGAGCGTAACCCCAGTGCGAAAGTTCCCGCCG
  361 ---------+---------+---------+---------+---------+---------+
      GAAATCGCAGCCACGTTACGCTCGTGGACATACCGATTTCGGCCCGGCCGCGGCGAGACG
  421 ---------+---------+---------+---------+---------+---------+
      ATAGGTTGTCGGGGTGACTGCCACAGCCACTGAAGGGGCCAAACCCCCATTCGTATCCCG
  481 ---------+---------+---------+---------+---------+---------+
                        V   T   A   T   A   T   E   G   A   K   P   P   F   V   S   R
      TTCAGTCCTGGTTACCGGAGGAAACCGGGGGATCGGGCTGGCGATCGCACAGCGGCTGGC
  541 ------+---------+---------+---------+---------+---------+---------+
       S   V   L   V   T   G   G   N   R   G   I   G   L   A   I   A   Q   R   L   A
      TGCCGACGGCCACAAGGTGGCCGTCACCCACCGTGGATCCGGAGCGCCAAAGGGGCTGTT
  601 ---------+---------+---------+---------+---------+---------+---------+
       A   D   G   H   K   V   A   V   T   H   R   G   S   G   A   P   K   G   L   F
      TGGCGTCGAATGTGACGTCACCGACAGCGACGCCGTCGATCGCGCCTTCACGGCGGTAGA
  661 ---------+---------+---------+---------+---------+---------+---------+
       G   V   E   C   D   V   T   D   S   D   A   V   D   R   A   F   T   A   V   E
      AGAGCACCAGGGTCCGGTCGAGGTGCTGGTGTCCAACGCCGGCCTATCCGCGGACGCATT
  721 ---------+---------+---------+---------+---------+---------+---------+
       E   H   Q   G   P   V   E   V   L   V   S   N   A   G   L   S   A   D   A   F
```

FIG. 9A

```
                CCTCATGCGGATGACCGAGGAAAAGTTCGAGAAGGTCATCAACGCCAACCTCACCGGGGC
781             ---------+---------+---------+---------+---------+---------+
                 L  M  R  M  T  E  E  K  F  E  K  V  I  N  A  N  L  T  G  A
                GTTCCGGGTGGCTCAACGGGCATCGCGCAGCATGCAGCGCAACAAATTCGGTCGAATGAT
841             ---------+---------+---------+---------+---------+---------+
                 F  R  V  A  Q  R  A  S  R  S  M  Q  R  N  K  F  G  R  M  I
                ATTCATAGGTTCGGTCTCCGGCAGCTGGGGCATCGGCAACCAGGCCAACTACGCAGCCTC
901             ---------+---------+---------+---------+---------+---------+
                 F  I  G  S  V  S  G  S  W  G  I  G  N  Q  A  N  Y  A  A  S
                CAAGGCCGGAGTGATTGGCATGGCCCGCTCGATCGCCCGCGAGCTGTCGAAGGCAAACGT
961             ---------+---------+---------+---------+---------+---------+
                 K  A  G  V  I  G  M  A  R  S  I  A  R  E  L  S  K  A  N  V
                GACCGCGAATGTGGTGGCCCCGGGCTACATCGACACCGATATGACCCGCGCGCTGGATGA
1021            ---------+---------+---------+---------+---------+---------+
                 T  A  N  V  V  A  P  G  Y  I  D  T  D  M  T  R  A  L  D  E
                GCGGATTCAGCAGGGGGCGCTGCAATTTATCCCAGCGAAGCGGGTCGGCACCCCCGCCGA
1081            ---------+---------+---------+---------+---------+---------+
                 R  I  Q  Q  G  A  L  Q  F  I  P  A  K  R  V  G  T  P  A  E
                GGTCGCCGGGGTGGTCAGCTTCCTGGCTTCCGAGGATGCGAGCTATATCTCCGGTGCGGT
1141            ---------+---------+---------+---------+---------+---------+
                 V  A  G  V  V  S  F  L  A  S  E  D  A  S  Y  I  S  G  A  V
                CATCCCGGTCGACGGCGGCATGGGTATGGGCCACTGACACAACACAAGGACGCACATGAC
1201            ---------+---------+---------+---------+---------+---------+
                 I  P  V  D  G  G  M  G  M  G  H  *                 M  T
                AGGACTGCTGGACGGCAAACGGATTCTGGTTAGCGGAATCATCACCGACTCGTCGATCGC
1261            ---------+---------+---------+---------+---------+---------+
                 G  L  L  D  G  K  R  I  L  V  S  G  I  I  T  D  S  S  I  A
```

FIG. 9B

```
                GTTTCACATCGCACGGGTAGCCCAGGAGCAGGGCGCCCAGCTGGTGCTCACCGGGTTCGA
1321            ---------+---------+---------+---------+---------+---------+

                 F  H  I  A  R  V  A  Q  E  Q  G  A  Q  L  V  L  T  G  F  D

                CCGGCTGCGGCTGATTCAGCGCATCACCGACCGGCTGCCGGCAAAGGCCCCGCTGCTCGA
1381            ---------+---------+---------+---------+---------+---------+

                 R  L  R  L  I  Q  R  I  T  D  R  L  P  A  K  A  P  L  L  E

                ACTCGACGTGCAAAACGAGGAGCACCTGGCCAGCTTGGCCGGCCGGGTGACCGAGGCGAT
1441            ---------+---------+---------+---------+---------+---------+

                 L  D  V  Q  N  E  E  H  L  A  S  L  A  G  R  V  T  E  A  I

                CGGGGCGGGCAACAAGCTCGACGGGGTGGTGCATGCGATTGGGTTCATGCCGCAGACCGG
1501            ---------+---------+---------+---------+---------+---------+

                 G  A  G  N  K  L  D  G  V  V  H  A  I  G  F  M  P  Q  T  G

                GATGGGCATCAACCCGTTCTTCGACGCGCCCTACGCGGATGTGTCCAAGGGCATCCACAT
1561            ---------+---------+---------+---------+---------+---------+

                 M  G  I  N  P  F  F  D  A  P  Y  A  D  V  S  K  G  I  H  I

                CTCGGCGTATTCGTATGCTTCGATGGCCAAGGCGCTGCTGCCGATCATGAACCCCGGAGG
1621            ---------+---------+---------+---------+---------+---------+

                 S  A  Y  S  Y  A  S  M  A  K  A  L  L  P  I  M  N  P  G  G

                TTCCATCGTCGGCATGGACTTCGACCCGAGCCGGGCGATGCCGGCCTACAACTGGATGAC
1681            ---------+---------+---------+---------+---------+---------+

                 S  I  V  G  M  D  F  D  P  S  R  A  M  P  A  Y  N  W  M  T

                GGTCGCCAAGAGCGCGTTGGAGTCGGTCAACAGGTTCGTGGCGCGCGAGGCCGGCAAGTA
1741            ---------+---------+---------+---------+---------+---------+

                 V  A  K  S  A  L  E  S  V  N  R  F  V  A  R  E  A  G  K  Y

                CGGTGTGCGTTCGAATCTCGTTGCCGCAGGCCCTATCCGGACGCTGGCGATGAGTGCGAT
1801            ---------+---------+---------+---------+---------+---------+

                 G  V  R  S  N  L  V  A  A  G  P  I  R  T  L  A  M  S  A  I
```

FIG. 9C

```
1861  GCTCGGCGGTGCGCTCGGCGAGGAGGCCGGCGCCCAGATCCAGCTGCTCGAGGAGGGCTG
      ---------+---------+---------+---------+---------+---------+

       V  G  G  A  L  G  E  E  A  G  A  Q  I  Q  L  L  E  E  G  W

1921  GGATCAGCGCGWTCCGATCGGCTGGAACATGAAGGATGCOACGCCGGTCGCCAAGACGGT
      ---------+---------+---------+---------+---------+---------+

       D  Q  R  A  P  I  G  W  N  M  K  D  A  T  P  V  A  K  T  V

1981  GTGCGCGCTGCTGTCTGACTGGCTGCCGGCGACCACGGGTGACATCATCTACGCCGACGG
      ---------+---------+---------+---------+---------+---------+

       C  A  L  L  S  D  W  L  P  A  T  T  G  D  I  I  Y  A  D  G

2041  CGGCGCGCACACCCAATTGCTCTAGAACGCATGCAATTTGATGCCGTCCTGCTGCTGTCG
      ---------+---------+---------+---------+---------+---------+

       G  A  H  T  Q  L  L  *

2101  TTCGGCGGACCGGAAGGGCCCGAGCAGGTGCGGCCGTTCCTGGAGAACGTTACCCGGGGC
      ---------+---------+---------+---------+---------+---------+

2161  CGCGGTGTGCCTGCCGAACGGTTGGACGCGGTGGCCGAGCACTACCTGCATTTCGGTGGG
      ---------+---------+---------+---------+---------+---------+

2221  GTATCACCGATC
      ---------+--
```

FIG. 9D

AMINO ACID SEQUENCE OF PS5 ENCODED BY NUCLEIC ACID RESIDUES 1256-2062

MTGLLDGKRI LVSGIITDSS IAFHIARYAQ EQGAQLVLTG FDRLRLIQRI

TDRLPQKAPL LELDVQNEEH LASLAGRVTE AIGAGNKLDG VVHAIGFMPQ

TGMGINPFFD APYADVSKGI HISAYSYASM AKALLPIMNP GGSIVGMDFD

PSRAMPAYNW MTVAKSALES VNRFVAREAG KYGVRSNLVA AGPIRTLAMS

AIVGGALGEE AGAQIQLLEE GWDQRAPIGW NMKDAIPVAK TVCALLSDWL

PATTGDIIYA DGGAHTQLL

FIG. 10

AMINO ACID SEQUENCE OF PS5 ENCODED BY NUCLEIC ACID RESIDUES 494-1234

VTATATEGAK PPFVSRSVLV TGGNRGIGLA IAQRLAADGH KVAVTHRGSG

APKGLFGVEC DVTDSDAVDR AFTAVEEHQG PVEVLVSNAG LSADAFLMRM

TEEKFEKVIN ANLTGAFRVA QRASRSMQRN KFGRMIFIGS VSGSWGIGNQ

ANYAASKAGV IGMARSIARE LSKANVTANV VAPGYIDTDM TRALDERIQQ

GALQFIPAKR VGTPAEVAGV VSFLASEDAS YISGAVIPVD GGMGMGH

FIG. 11

DNA Sequence of pS5

```
    GTTCGCTCCG GCGCGGTCAC GCGCATGCCC TCGATGACGC AGATCTCGTC
 51 GGGCTCGATG CGCTCTTCCC AGACTTGCAG CCCCGGGGCA CGGCGGCGGT
101 TGGTGTCGAT GATCGCGGCG GGAAGATCCG CGTCGATCCA CTTGGCGCCA
151 TGGAAGGCAG AAGCCGAGTA GCCGGCCAGC ACGCCGCGGC GGCGCGAGCG
201 CAGCCACAGC GCTTTTGCAC GCAATTGCGC GGTCAGTTCC ACACCCTGCG
251 GCACGTACAC GTCTTTATGT AGCGCGACAT ACCTGCTGCG CAATTCGTAG
301 GGCGTCAATA CACCCGCAGC CAGGGCCTCG CTGCCCAGAA AGGGATCCGT
351 CATGGTCGAA GTGTGCTGAG TCACACCGAC AAACGTCACG AGCGTAACCC
401 CAGTGCGAAA GTTCCCGCCG GAAATCGCAG CCACGTTACG CTCGTGGACA
451 TACCGATTTC GGCCCGGCCG CGGCGAGACG ATAGGTTGTC GGGGTGACTG
501 CCACAGCCAC TGAAGGGGCC AAACCCCCAT TCGTATCCCG TTCAGTCCTG
551 GTTACCGGAG GAAACCGGGG GATCGGGCTG GCGATCGCAC AGCGGCTGGC
601 TGCCGACGGC CACAAGGTGG CCGTCACCCA CCGTGGATCC GGAGCGCCAA
651 AGGGGCTGTT TGGCGTCGAA TGTGACGTCA CCGACAGCGA CGCCGTCGAT
701 CGCGCCTTCA CGGCGGTAGA AGAGCACCAG GGTCCGGTCG AGGTGCTGGT
751 GTCCAACGCC GGCCTATCCG CGGACGCATT CCTCATGCGG ATGACCGAGG
801 AAAAGTTCGA GAAGGTCATC AACGCCAACC TCACCGGGGC GTTCCGGGTG
851 GCTCAACGGG CATCGCGCAG CATGCAGCGC AACAAATTCG GTCGAATGAT
901 ATTCATAGGT TCGGTCTCCG GCAGCTGGGG CATCGGCAAC CAGGCCAACT
```

FIG. 12A

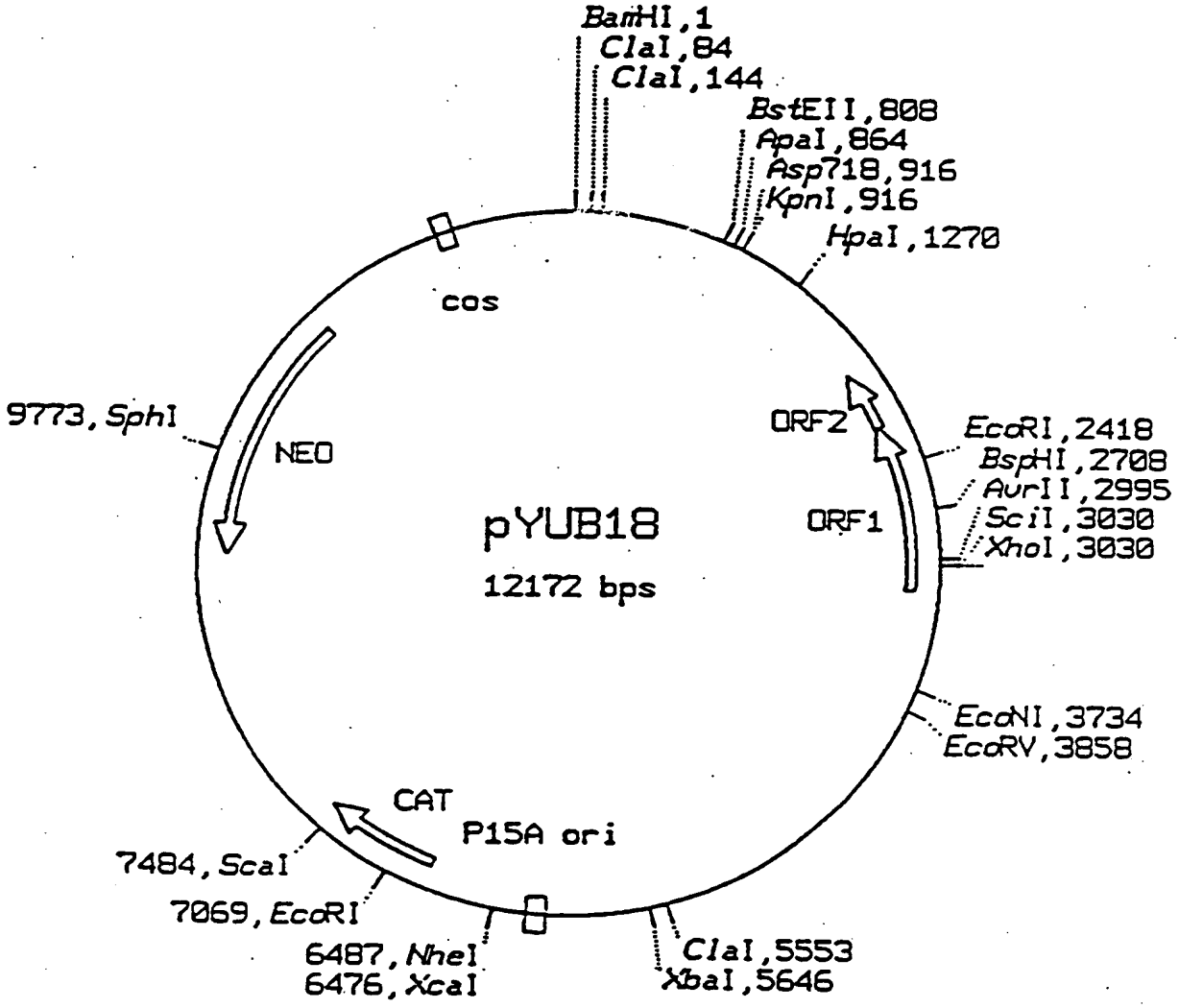
```
 951 ACGCAGCCTC CAAGGCCGGA GTGATTGGCA TGGCCCGCTC GATCGCCCGC
1001 GAGCTGTCGA AGGCAAACGT GACCGCGAAT GTGGTGGCCC CGGGCTACAT
1051 CGACACCGAT ATGACCCGCG CGCTGGATGA GCGGATTCAG CAGGGGGCGC
1101 TGCAATTTAT CCCAGCGAAG CGGGTCGGCA CCCCCGCCGA GGTCGCCGGG
1151 GTGGTCAGCT TCCTGGCTTC CGAGGATGCG AGCTATATCT CCGGTGCGGT
1201 CATCCCGGTC GACGGCGGCA TGGGTATGGG CCACTGACAC AACACAAGGA
1251 CGCACATGAC AGGACTGCTG GACGGCAAAC GGATTCTGGT TAGCGGAATC
1301 ATCACCGACT CGTCGATCGC GTTTCACATC GCACGGGTAG CCCAGGAGCA
1351 GGGCGCCCAG CTGGTGCTCA CCGGGTTCGA CCGGCTGCGG CTGATTCAGC
1401 GCATCACCGA CCGGCTGCCG GCAAAGGCCC CGCTGCTCGA ACTCGACGTG
1451 CAAAACGAGG AGCACCTGGC CAGCTTGGCC GGCCGGGTGA CCGAGGCGAT
1501 CGGGGCGGGC AACAAGCTCG ACGGGGTGGT GCATGCGATT GGGTTCATGC
1551 CGCAGACCGG GATGGGCATC AACCCGTTCT TCGACGCGCC CTACGCGGAT
1601 GTGTCCAAGG GCATCCACAT CTCGGCGTAT TCGTATGCTT CGATGGCCAA
1651 GGCGCTGCTG CCGATCATGA ACCCCGGAGG TTCCATCGTC GGCATGGACT
1701 TCGACCCGAG CCGGGCGATG CCGGCCTACA ACTGGATGAC GGTCGCCAAG
1751 AGCGCGTTGG AGTCGGTCAA CAGGTTCGTG GCGCGCGAGG CCGGCAAGTA
1801 CGGTGTGCGT TCGAATCTCG TTGCCGCAGG CCCTATCCGG ACGCTGGCGA
```

FIG. 12B

```
1851 TGAGTGCGAT CGTCGGCGGT GCGCTCGGCG AGGAGGCCGG CGCCCAGATC
1901 CAGCTGCTCG AGGAGGGCTG GGATCAGCGC GCTCCGATCG GCTGGAACAT
1951 GAAGGATGCG ACGCCGGTCG CCAAGACGGT GTGCGCGCTG CTGTCTGACT
2001 GGCTGCCGGC GACCACGGGT GACATCATCT ACGCCGACGG CGGCGCGCAC
2051 ACCCAATTGC TCTAGAACGC ATGCAATTTG ATGCCGTCCT GCTGCTGTCG
2101 TTCGGCGGAC CGGAAGGGCC CGAGCAGGTG CGGCCGTTCC TGGAGAACGT
2151 TACCCGGGGC CGCGGTGTGC CTGCCGAACG GTTGGACGCG GTGGCCGAGC
2201 ACTACCTGCA TTTCGGTGGG GTATCACCGA TC
```

FIG. 12C

FIGURE 13


BamHI, 1
ClaI, 84
ClaI, 144
BstEII, 808
ApaI, 864
Asp718, 916
KpnI, 916
HpaI, 1270
cos
9773, SphI
NEO
ORF2
EcoRI, 2418
BspHI, 2708
AvrII, 2995
SciI, 3030
XhoI, 3030
pYUB18
12172 bps
ORF1
EcoNI, 3734
EcoRV, 3858
CAT
P15A ori
7484, ScaI
7069, EcoRI
6487, NheI
6476, XcaI
ClaI, 5553
XbaI, 5646

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 48/00; C07H 21/00; C12P 19/34

US CL :536/23.2; 514/44; 435/91

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.2; 514/44; 435/91

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Science, Volume 263, issued 14 January 1994, A. Banerjee et al., "*inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*", pages 227-230, see entire article.	1-7
Y	Chemical Reviews, Volume 90, No. 4, issued June 1990, E. Uhlmann et al., "Antisense oligonucleotides: A new therapeutic principle", pages 543-584, see entire article.	8, 9
Y	Genomics, Volume 5, issued 1989, M. Orita et al., "Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction", pages 874-879, see entire article.	10-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 02 AUGUST 1994	Date of mailing of the international search report 19 AUG 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Suzanne Ziska, Ph.D. R. Kruza for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Clinical Microbiology, Volume 31, No. 2, issued February 1993, A. Telenti et al., " Rapid identification of Mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis", pages 175-178, see entire article.	10-13

Box I Observations where certain claims were f und unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-13

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ N protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-9, claims 1-7 drawn to a first product, a gene, and claims 8 and 9, drawn to a first method of using th first product, a method of treating an individual, classified in Class 536, subclass 23.2, and Class 514, subclass 44, for example.

Group II, claims 10-13, drawn to a second method, a method of assessing susceptibility of a strain of mycobacteria in a biological sample to INH, classified in Class 435, subclass 91, for example.

Group III, claim 14, drawn to a third method, a method of determining whether a drug is effective against mycobacterial infection, classified in Class 437, subclass 7.7, for example.

Group IV, claim 15, drawn to a fourth method, a method for producing a tuberculosis specific mycolic acid comprising adding purified InhA to substrates, classified in Class 435, subclass 41, for example.

Group V, claim 16, drawn to a fifth method, a method for producing a compound that inhibits InhA activity, classified in Class 435, subclass 7.72, for example.

Group VI, claim 17, drawn to a second product, an isolated inhA polypeptide, classified in Class 530, subclass 350, for example.
Group VII, claim 18, a third product, a vaccine, classified in Class 424, subclass 93D, for example.

Each of the products in an independent and distinct product since polypeptides are materially different than nucleic acids (genes) and both are materially different than vaccines. Each of the methods is an independent and distinct method since the methods involve different procedures, the creation of different products. Each grouping of claims forms a separate invention not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.